Consejo Superior de Investigaciones Científicas
Real Jardín Botánico de Madrid

Universidad Autónoma de Madrid
Facultad de Ciencias
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Evolución en el género *Linaria*: biogeografía, cambio morfológico y sistemática de *Linaria* sect. *Versicolores*

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Memoria de Tesis Doctoral

Madrid, 2012

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
REAL JARDÍN BOTÁNICO DE MADRID

UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS
DEPARTAMENTO DE BIOLOGÍA

EVOLUCIÓN EN EL GÉNERO *LINARIA*: BIOGEOGRAFÍA, CAMBIO MORFOLÓGICO Y SISTEMÁTICA DE *LINARIA* SECT. *VERSICOLORES*

Memoria para optar al grado de Doctor en Biología que presenta

Mario Fernández-Mazuecos Santa Teresa

Director
Pablo Vargas Gómez

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Madrid, octubre de 2012

Fotografías de la cubierta, de izquierda a derecha y de arriba a abajo: *Linaria viscosa* subsp. *viscosa* (José Quiles), *L. clementei* (Joaquín Ramírez), *L. tenuis* (Ori Fragman-Sapir), *L. nigricans* (Pablo Vargas) y *L. onubensis* (Pablo Vargas).

Fotografía del lomo: *L. elegans* (Pablo Vargas).

Imagen de la contracubiera: región Mediterránea visualizada en el *software* Celestia (http://www.shatters.net/celestia/).

Agradecimientos

A mi director de tesis, Pablo Vargas, por darme la oportunidad de trabajar en su grupo de investigación, por su dedicación, buenas ideas y motivación permanente.

A todo su grupo de investigación, por su compañía y apoyo permanente en este viaje, y por los divertidos momentos brindados. En particular, a Emilio Cano por sus enseñanzas en el laboratorio y por su eficacia en la resolución de problemas técnicos. A José Luis Blanco, "hermano científico" en el estudio de las linarias, por su ayuda permanente en todos los aspectos y por la interesante (e interminable) discusión que me ha proporcionado. A Isabel Liberal, Pedro Jiménez Mejías y Elena Amat, por su ayuda en distintos campos y por su interés en el desarrollo de esta tesis. A Luis Valente y Beatriz Guzmán, por su asistencia en el aprendizaje de algunas de las técnicas más difíciles; sus tesis, además, sirvieron como modelos de rigor científico a seguir en el desarrollo de ésta. También a los que pasaron por el grupo de forma temporal: Marisa Navarro, Beatriz Rumeu, José Ruiz, entre otros. A Beatriz Vigalondo, por su productiva colaboración en el estudio sistemático del complejo *Linaria incarnata*.

A mi tutor académico, Juan Carlos Moreno, por su atención e interés, y por su eficacia en la resolución de cuestiones burocráticas.

A todos los investigadores que, en mayor o menor medida, han participado, colaborado o ayudado en el desarrollo de esta tesis. En particular, a Enrico Coen y Xana Rebocho por acogerme en su laboratorio durante mi estancia en el John Innes Centre de Norwich. A José María Gómez por su colaboración e interesante discusión en el capítulo de evolución floral, y por acogerme durante mi estancia en el departamento de Ecología de la Universidad de Granada. A Llorenç Sáez por su colaboración y productiva discusión en el aspecto taxonómico. A Ana Juan, por su orientación al inicio de esta tesis. A Jesús Muñoz, por su orientación en la modelización de la distribución de *Linaria elegans*. A Concepción Ornosa, Roger Vila, Miguel Carles-Tolrá y Javier Ortiz por su ayuda en la identificación de los insectos polinizadores. Y a todo el profesorado del curso de Filogenias y Genealogías de ADN de la Universidad de Barcelona, por sus enseñanzas esenciales para mi aprendizaje de los análisis que constituyen el grueso de esta tesis.

A todo el personal técnico del laboratorio del Real Jardín Botánico de Madrid: Fátima Durán, Guillermo Sanjuanbenito, Yolanda Ruiz y Gemma Andreu, por la inestimable ayuda técnica proporcionada.

Al personal de los distintos herbarios que proporcionaron especímenes de *Linaria* esenciales para el desarrollo de esta tesis. En especial, al herbario del Real Jardín Botánico de Madrid, a su conservador Mauricio Velayos por la facilitación de los permisos para la obtención de material para la extracción de ADN, y a su personal, en particular a Concha Baranda y Charo Noya, por la ayuda prestada en la tramitación de préstamos y el manejo del material. También agradecemos especialmente al herbario de la Universidad de Reading, a su *curator*, Stephen Jury, y a su *deputy curator*, Ronald Rutherford, por el préstamo de abundante material y el permiso para la extracción de ADN de los pliegos, lo que ha resultado fundamental para la inclusión en nuestra investigación de especies y poblaciones de *Linaria* del norte de África. También a los herbarios de la Universidad Pablo de Olavide de Sevilla, la Universidad de Sevilla, el Museo de Historia Natural Goulandris de Atenas, la Universidad de Salamanca y el Real Jardín Botánico de Edimburgo por su aportación de material. A Alan Forrest por facilitarnos los trámites del préstamo de material de éste último.

A todos los que proporcionaron material de *Linaria* de sus propias recolecciones: Francisco Gómiz, Enrique Sánchez-Gullón, Santiago Martín-Bravo, Juanjo Aldasoro, Marisa Alarcón, Elena Amat, Belén Estébanez, Nagore García, Jaime Güemes, Juan Carlos Moreno, Enrique Rico, Pedro Jiménez Mejías, Juancho Calleja y Shuji Taniguchi.

A las numerosas personas que facilitaron la localización de poblaciones de *Linaria* en la península Ibérica. Enrique Sánchez-Gullón en la provincia de Huelva, Joaquín Ramírez en Málaga, y Bernardo García en Ávila aportaron su extraordinario conocimiento de la distribución de las poblaciones, y proporcionaron, además, asistencia en el trabajo de campo. Javier Amigo, Carlos Molina, José Luis Benito, Juan José Sánchez, Gonzalo Mateo y Ginés López ayudaron en la localización de poblaciones de *Linaria elegans*.

A todos aquellos, compañeros, amigos y familiares, que también echaron una mano en el trabajo de campo y me permitieron llegar, mediante su pericia automovilística, a los lugares

de muestreo: Alberto Bañón, José Luis Blanco, Fidel y Alberto Fernández-Mazuecos, Belén Estébanez, David Orgaz, Juan Carlos Moreno y Javier Freijanes.

A aquellos que cedieron desinteresadamente sus brillantes fotografías para ilustrar esta memoria: José Quiles, Joaquín Ramírez, Modesto Luceño, Ori Fragman-Sapir, Nick Kurzenko y Gerald D. Carr.

Al personal de la Biblioteca del Real Jardín Botánico de Madrid, y en especial a Helena Velayos, por la asistencia prestada para la búsqueda bibliográfica y los préstamos interbibliotecarios.

Al Consejo Superior de Investigaciones Científicas y al Ministerio de Ciencia e Innovación del Gobierno de España, que financiaron mi trabajo a través de sendas becas predoctorales (JAE Predoctoral y FPU –referencia AP2007-01841– respectivamente) y distintos proyectos de investigación, particularmente el proyecto "Evolución de la flor personada" (CGL2009-10031, Ministerio de Ciencia e Innovación, Plan Nacional I+D).

A todos los compañeros y amigos del Real Jardín Botánico de Madrid. Además de los mencionados más arriba, también a Eva, Raquel, Juan Carlos, Arantxa, Andrea, Irene, Marieta, Melissa, Alberto, Alejandro, Mario Mairal, Vladi, Leopoldo, Maxi, Joel, Jano, Alicia, Li-Fang, Isabel Marques… También a los que hicieron más divertida mi estancia en Norwich: Panos, Yara y Verónica.

A mis amigos, en especial a aquellos que siguieron un poco más de cerca el desarrollo de esta tesis: David, Alberto, Lali, Belén, Salvador, Laura, Javi, Araceli.

A Ale, por su apoyo, paciencia y comprensión constantes.

A mis padres, Fidel y Mª Ángeles, y a mis hermanos, Alberto y Alicia, sin cuyo apoyo no habría iniciado este camino ni habría llegado hasta aquí.

A todos, ¡GRACIAS!

Índice

Resumen

Summary

Toadflaxes (*Linaria* Mill., c. 150 species) constitute the most diverse genus within the tribe Antirrhineae (Plantaginaceae). Here we present the first detailed evolutionary analysis of toadflaxes, with special emphasis on bifid toadflaxes (*Linaria* sect. *Versicolores* (Benth.) Wettst.), a distinctive assemblage of c. 25 species mainly distributed in the western Mediterranean region. Hypotheses concerning biogeographic patterns, morphological trait evolution and systematics are tested by applying molecular phylogenetic and phylogeographic methods at different macro- and microevolutionary levels. The analysis of both nuclear and plastid DNA markers is accompanied by morphometric analyses of flower shape, species distribution modelling and taxonomic revisions. The integration of different data sources has enabled a robust reconstruction of the evolution of bifid toadflaxes in a spatio-temporal framework. The group has been confirmed to be monophyletic, and its most recent common ancestor has been dated back to the late Miocene or Pliocene. According to our results, the evolution of bifid toadflaxes has been significantly affected by both historical abiotic factors (the history of contacts and isolation between the Eurasian and African plates, the onset of the Mediterranean climate, the Quaternary climatic cycles) and biotic factors (insect pollinators). The contribution and prevalence of each factor in the section as a whole or in particular lineages is assessed.

Capítulo 1

Introducción

La biología evolutiva ha experimentado un notable avance en las últimas décadas con el desarrollo de las técnicas de secuenciación de ADN y de un gran número de herramientas para el análisis de secuencias en el marco de la sistemática filogenética (Felsenstein, 2004; Lemey *et al.*, 2009). Esta aproximación a la biología evolutiva viene a complementar a otras disciplinas que tienen un origen más antiguo, pero que también se han visto beneficiadas por los avances tecnológicos y analíticos, como la taxonomía y la sistemática basadas en caracteres morfológicos, la paleontología, la citogenética, la biología reproductiva y la ecología (Stuessy, 2009). La integración de la información procedente de estas disciplinas con las inferencias de relaciones filogenéticas basadas en secuencias de ADN permite una reconstrucción cada vez más fiable de los patrones evolutivos de todo tipo de organismos en un contexto espacial y temporal (Lecointre & Le Guyader, 2006; Hedges & Kumar, 2009). Asimismo, la aplicación de métodos filogenéticos comparativos, la realización de experimentos de selección natural en poblaciones actuales y el estudio comparativo del desarrollo a lo largo de la evolución (en la disciplina denominada *evo-devo*) ayudan a la comprensión de los procesos evolutivos que subyacen a esos patrones (Harvey & Pagel, 1991; Gould, 2002; Carroll, 2006; Futuyma, 2009; Nunn, 2011). De este modo, hoy podemos comprender el cambio evolutivo con una profundidad y un detalle impensables cuando Charles Darwin y Alfred Russel Wallace propusieron por primera vez su teoría de la evolución por selección natural.

Dentro de las plantas con flores, la tribu Antirrhineae (Plantaginaceae) constituye un grupo de organismos con una larga tradición en el estudio de su evolución, sistemática, morfología, desarrollo, ecología y otros aspectos de su biología (Linnaeus, 1749; Darwin, 1868; Sutton, 1988; Schwarz-Sommer *et al.*, 2003; Vargas *et al.*, 2004). El género *Linaria* es, con unas 150 especies, el más diverso de este grupo y, sin embargo, apenas se conocen sus relaciones de parentesco infragenéricas. Con la presente memoria doctoral se dará el primer paso para subsanar esa laguna de conocimiento, y se sentarán las bases para el análisis evolutivo de un género de innegable interés debido a su diversidad, a sus caracteres florales y a sus patrones biogeográficos.

La tribu Antirrhineae (Plantaginaceae)

La tribu Antirrhineae está constituida por unos una treintena de géneros distribuidos por la región Paleártica y América (Sutton, 1988; Vargas *et al.*, 2004). Es un grupo morfológicamente bien definido dentro del clado al que pertenece (véase más abajo) por la dehiscencia poricida de sus cápsulas (Sutton, 1988). También se caracteriza por la presencia de un glucósido iridoide casi exclusivo del grupo, el antirrhinósido (Kooiman, 1970; Nicoletti *et al.*, 1988; Albach *et al.*, 2005), y por el desarrollo inicial de haustorios en el endospermo (Sutton, 1988). Las flores son gamopétalas, generalmente zigomorfas y frecuentemente personadas (Sutton, 1988). Históricamente se ha considerado que las antirrhineas forman un grupo natural (Chavannes, 1833; Sutton, 1988), y los análisis filogenéticos basados en secuencias de ADN efectuados en los últimos años han venido a confirmarlo (Vargas *et al.*, 2004; Albach *et al.*, 2005). El estudio evolutivo de la tribu Antirrhineae es de gran interés, entre otros motivos por el hecho de incluir el género *Antirrhinum*, un organismo modelo para la genética y la biología del desarrollo (Schwarz-Sommer *et al.*, 2003). Esto permite la realización de estudios de biología evolutiva del desarrollo (*evo-devo*) que difícilmente son posibles en otros linajes de plantas (e.g. Hileman *et al.*, 2003; Feng *et al.*, 2009; Box *et al.*, 2011). A continuación se repasan brevemente algunos aspectos del grupo que son relevantes para la presente memoria doctoral.

Adscripción taxonómica

La adscripción familiar de las antirrhineas es controvertida. Tradicionalmente se han incluido en la familia Scrophulariaceae. Recientemente, sin embargo, los análisis filogenéticos han venido a "desintegrar" esta familia en su sentido clásico, dividiéndola en un número variable de familias correspondientes a grupos monofiléticos dentro del orden Lamiales (Olmstead & Reeves, 1995; Olmstead *et al.*, 2001; Oxelman *et al.*, 2005; Schäferhoff *et al.*, 2010). De acuerdo con esta aproximación, la tribu Antirrhineae ha quedado integrada en un clado morfológicamente muy variado, para el cual se propusieron los nombres de Antirrhinaceae (Reveal *et al.*, 1999) y Veronicaceae (Olmstead *et al.*, 2001), para finalmente denominarse Plantaginaceae (APG II, 2003; Albach *et al.*, 2005; Oxelman *et al.*, 2005; APG III, 2009) como consecuencia de la prioridad nomenclatural de este nombre. Algunos autores, sin embargo, se han mostrado partidarios de

seguir reconociendo la familia clásica Scrophulariaceae (*sensu lato*) como grupo parafilético (Heywood *et al.*, 2007) al tiempo que, para añadir más confusión, estos y otros autores continúan utilizando el nombre Plantaginaceae (*sensu stricto*) para referirse al linaje de *Plantago* y géneros relacionados (e.g. Hoggard *et al.*, 2003). En realidad, la familia Plantaginaceae *sensu lato* integra un conjunto demasiado heterogéneo de plantas y carece, por el momento, de sinapomorfías morfológicas claras que sirvan como caracteres diagnósticos para su identificación (Albach *et al.*, 2005), aunque Judd *et al.* (2002) han señalado la ausencia de particiones verticales en los pelos como posible sinapomorfía. Nosotros, en cualquier caso, consideraríamos más apropiada una aproximación analítica a la sistemática de este clado, en la que se reconocieran como familias grupos monofiléticos con caracteres diagnósticos (sinapomorfías) bien definidos. Según este punto de vista, los treinta géneros de antirrhineas podrían reconocerse como una familia independiente, Antirrhinaceae. En cualquier caso, se requiere aún la inclusión de un mayor número de géneros en los análisis filogenéticos (Fischer, 2004), así como el análisis de más regiones de ADN (particulamente nucleares), antes de establecer una clasificación definitiva de este clado. Dada la situación taxonómica aún no sólida, en la presente memoria doctoral hemos optado por mantenernos al margen del problema y adscribir el género *Linaria* a un grupo clásicamente definido desde el punto de vista morfológico y bien apoyado desde el punto de vista filogenético: la tribu Antirrhineae.

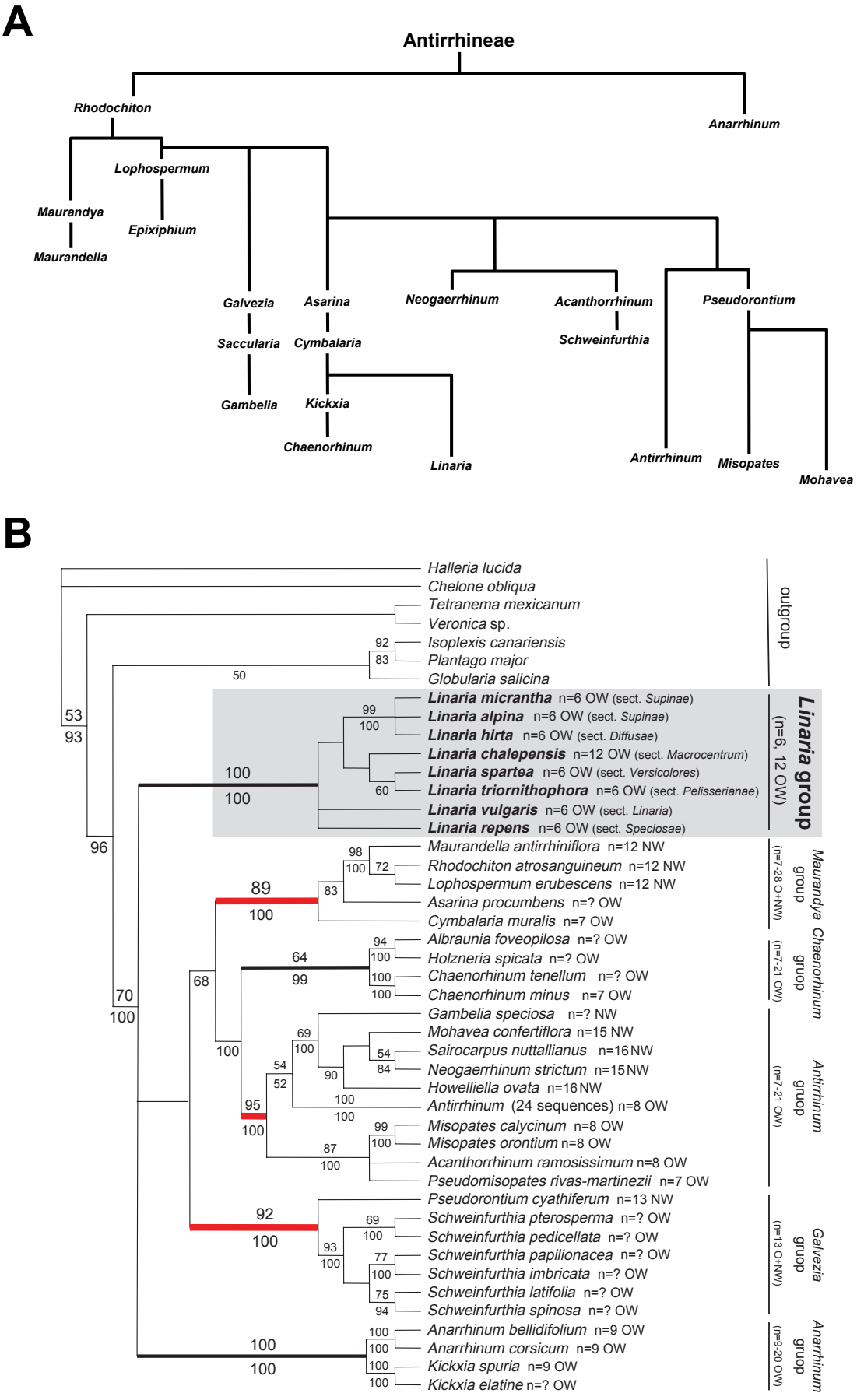
Sistemática, biogeografía y filogenia

Linneo (1753) incluyó 28 especies consideradas hoy día como antirrhineas en el género *Antirrhinum*. Posteriormente se fue adoptando una circunscripción más restringida de *Antirrhinum* y se reconoció un número creciente de géneros (véase Miller, 1754), siguiendo en algunos casos las propuestas acertadas de taxónomos prelinneanos como Tournefort (1700). La primera monografía de la tribu se debe a Chavannes (1833), que reconoció 107 especies en seis géneros. Tras las importantes aportaciones de Bentham (1846), Wettstein (1895) y Rothmaler (1943, 1956), la sistemática y la taxonomía de la tribu Antirrhineae fueron revisadas por Sutton (1988) en una exhaustiva y magníficamente documentada síntesis que reconoció un total de 326 especies y 27 géneros (Tabla 1). En comparación con tratamientos anteriores, Sutton aportó la novedad de reconocer como géneros distintos varios conjuntos de especies

Tabla 1. Géneros de Antirrhineae según Sutton (1988). Se indican el número de especies reconocidas por el mismo autor y la distribución geográfica de cada género.

Género	Nº de especies	Distribución
Acanthorrhinum Rothm.	1	N de África.
Albraunia Speta	3	SO de Asia.
Anarrhinum Desf.	8	S y O de Europa, N de África, SO de Asia.
Antirrhinum L.	20	Región Mediterránea, principalmente la península Ibérica. Ampliamente naturalizado en regiones templadas.
Asarina Mill.	1	SO de Europa.
Chaenorhinum (DC.) Rchb.	21	Principalmente SO de Europa, N de África y SO de Asia. Ampliamente naturalizado en regiones templadas.
Cymbalaria Hill	9	N del Mediterráneo, S de los Alpes. Naturalizado por el SO y C de Europa, ocasionalmente en otros lugares.
Epixiphium (A.Gray) Munz.	1	SO de Norteamérica.
Galvezia Domb. ex Juss.	4	Perú, Ecuador, islas Galápagos.
Gambelia Nutt.	4	SO de Norteamérica.
Holmgrenanthe Elisens	1	SO de Norteamérica.
Holzneria Speta	2	SO de Asia.
Howelliella Rothm.	1	SO de Norteamérica.
Kickxia Dumort.	46	Macaronesia, N de África, Europa, SC y SO de Asia. Ampliamente naturalizado en regiones templadas.
Linaria Mill.	150	Europa, excepto el extremo N; Asia excepto el SE y el extremo N; N de África. Frecuentemente introducido y naturalizado en otros lugares en regiones templadas.
Lophospermum D.Don	8	Norte y Centroamérica.
Mabrya Elisens	5	N de Méjico, SO de EE.UU.
Maurandella (A.Gray) Rothm.	1	SO de Norteamérica.
Maurandya Ortega	2	Norte y Centroamérica.
Misopates Raf.	7	Europa, N de África, Macaronesia, SO a SC de Asia.
Mohavea A.Gray	2	SO de Norteamérica.
Neogaerrhinum Rothm.	2	SO de Norteamérica.
Nuttallanthus D.A.Sutton	4	Norteamérica y Suramérica principalmente en el O. Naturalizado en otros lugares en regiones templadas.
Pseudorontium (A.Gray) Rothm.	1	SO de Norteamérica.
Rhodochiton Zucc.	3	C de Méjico.
Sairocarpus D.A.Sutton	13	SO de Norteamérica.
Schweinfurthia A.Braun	6	NE y CE de África, SO de Asia (especialmente la península Arábiga).

Fig. 1. Hipótesis filogenéticas de la tribu Antirrhineae. (A) Hipótesis de Rothmaler (1943), basada en caracteres morfológicos. (B) Análisis filogenético de Vargas *et al.* (2004), basado en secuencias nucleares de la región ITS; se muestra el árbol consenso de un análisis de parsimonia, con valores de *bootstrap* y probabilidad posterior bayesiana (en porcentaje) indicados encima y debajo de las ramas respectivamente; se indica el número cromosómico y la distribución de cada taxon (OW, Viejo Mundo; NW, Nuevo Mundo); se indican los seis clados principales de Antirrhineae y, en rojo, los tres linajes con disyunción Viejo Mundo-Nuevo Mundo.


A
Antirrhineae
Rhodochiton
Anarrhinum
Lophospermum
Maurandya
Epixiphium
Maurandella
Galvezia
Asarina
Neogaerrhinum
Acanthorrhinum
Pseudorontium
Saccularia
Cymbalaria
Schweinfurthia
Gambelia
Kickxia
Chaenorhinum
Linaria
Antirrhinum
Misopates
Mohavea
B
Halleria lucida
Chelone obliqua
Tetranema mexicanum
Veronica sp.
Isoplexis canariensis
Plantago major
Globularia salicina
outgroup
Linaria micrantha n=6 OW (sect. Supinae)
Linaria alpina n=6 OW (sect. Supinae)
Linaria hirta n=6 OW (sect. Diffusae)
Linaria chalepensis n=12 OW (sect. Macrocentrum)
Linaria spartea n=6 OW (sect. Versicolores)
Linaria triornithophora n=6 OW (sect. Pelisserianae)
Linaria vulgaris n=6 OW (sect. Linaria)
Linaria repens n=6 OW (sect. Speciosae)
Linaria group (n=6, 12 OW)
Maurandella antirrhiniflora n=12 NW
Rhodochiton atrosanguineum n=12 NW
Lophospermum erubescens n=12 NW
Asarina procumbens n=? OW
Cymbalaria muralis n=7 OW
Maurandya group (n=7-28 O+NW)
Albraunia foveopilosa n=? OW
Holzneria spicata n=? OW
Chaenorhinum tenellum n=? OW
Chaenorhinum minus n=7 OW
Chaenorhinum group (n=7-21 OW)
Gambelia speciosa n=? NW
Mohavea confertiflora n=15 NW
Sairocarpus nuttallianus n=16 NW
Neogaerrhinum strictum n=15 NW
Howelliella ovata n=16 NW
Antirrhinum (24 sequences) n=8 OW
Misopates calycinum n=8 OW
Misopates orontium n=8 OW
Acanthorrhinum ramosissimum n=8 OW
Pseudomisopates rivas-martinezii n=7 OW
Antirrhinum group (n=7-21 OW)
Pseudorontium cyathiferum n=13 NW
Schweinfurthia pterosperma n=? OW
Schweinfurthia pedicellata n=? OW
Schweinfurthia papilionacea n=? OW
Schweinfurthia imbricata n=? OW
Schweinfurthia latifolia n=? OW
Schweinfurthia spinosa n=? OW
Galvezia group (n=13 O+NW)
Anarrhinum bellidifolium n=9 OW
Anarrhinum corsicum n=9 OW
Kickxia spuria n=9 OW
Kickxia elatine n=? OW
Anarrhinum group (n=9-20 OW)

americanas que anteriormente otros autores habían incluido en los géneros principalmente paleárticos *Antirrhinum*, *Asarina* y *Linaria* (Tabla 1). En general, la delimitación genérica de Sutton se acepta en la actualidad, aunque con reticencias entre los botánicos americanos (véase Thompson, 1988; Oyama & Baum, 2004). Además, en los últimos tiempos se han reconocido dos géneros más: el monotípico *Pseudomisopates* (Güemes, 1997) y *Nanorrhinum*, que Ghebrehiwet (2000) escindió de *Kickxia*.

La disyunción anfiatlántica de las antirrhineas les confiere un interés biogeográfico notable (Raven & Axelrod, 1978; Hong, 1983). Se propuso la hipótesis de que habrían formado parte de una paleoflora esclerófila "Madreo-Tetiana" que habría ocupado de manera continua áreas de Norteamérica y Eurasia con sequía temporal a principios del Terciario, antes de la apertura del océano Atlántico (Axelrod, 1975; Raven & Axelrod, 1978). Posteriormente, con el establecimiento del clima mediterráneo en el oeste de Norteamérica (California) y en la cuenca del Mediterráneo (Suc, 1984; Millar, 2012) los linajes de antirrhineas habrían evolucionado ante esas nuevas condiciones separadamente en las dos regiones.

Rothmaler (1943) fue el primero en proponer explícitamente una hipótesis acerca de las relaciones filogenéticas de los géneros de Antirrhineae a partir de información morfológica (Fig. 1A). En esta propuesta, sugirió un estrecho parentesco entre todos los géneros que presentan en la corola un espolón portador de néctar: *Linaria*, *Cymbalaria*, *Kickxia* y *Chaenorhinum*. Elisens & Tomb (1983), a partir de caracteres de las semillas, presentaron una hipótesis acerca de las relaciones evolutivas entre los géneros del Nuevo Mundo, mientras que Ghebrehiwet (2000) efectuó un análisis cladístico basado en diversos caracteres morfológicos reproductivos y vegetativos en el que obtuvo escasa resolución. Con el desarrollo de las técnicas de secuenciación de ADN en los últimos años, se han podido poner a prueba, en parte, las hipótesis sistemáticas, biogeográficas y filogenéticas anteriores. Se han publicado análisis filogenéticos basados en secuencias plastidiales (Ghebrehiwet *et al.*, 2000) y nucleares (Vargas *et al.*, 2004) que han producido resultados relativamente congruentes, aunque el muestreo es aún incompleto y las relaciones entre los grandes clados no están totalmente resueltas. La filogenia más completa publicada hasta el momento es la de Vargas *et al.* (2004), basada en secuencias del espaciador transcrito interno (ITS) del ADN ribosómico nuclear (Fig. 1B). En ella se reconocieron seis clados principales, tres de ellos formados por géneros tanto del Viejo como del Nuevo Mundo (Fig. 1B),

lo que implicaría un mínimo de tres disyunciones independientes a través del Atlántico, que podrían haberse originado en el mismo marco temporal (Vargas *et al.*, 2004). Se ha propuesto que éste podría ser el primer caso de congruencia biogeográfica dentro de un mismo grupo (Vargas *et al.*, 2004; Wen & Ickert-Bond, 2009), aunque no se han aplicado aún técnicas de reloj molecular para la datación rigurosa de dichas divergencias a partir de calibraciones con fósiles. La posible conexión de las antirrhineas con una hipotética flora Madreo-Tetiana no ha sido, por tanto, evaluada. Este es el objetivo del trabajo incluido en el Apéndice 1 de esta memoria.

El género *Linaria* Mill.

Antecedentes históricos

Las linarias[1] constituyen el género más diverso (en número de especies y en disparidad morfológica) de la tribu Antirrhineae. Son plantas conocidas desde antiguo. El nombre parece proceder del latín medieval *linaria*, que tendría su origen en el parecido de sus hojas con las del lino (*Linum usitatissimum* L.) (Bauhin, 1623; Sáez & Bernal, 2009). Se ha especulado históricamente que la *osyris* de la Antigüedad, mencionada por Dioscórides en su *De materia medica* y por Plinio el Viejo en su *Naturalis historia*, corresponde a *Linaria vulgaris*, precisamente por el parecido indicado por estos autores entre las hojas de *osyris* y las del lino (Fuchs, 1542; Turner, 1568; Suárez de Ribera, 1733). Las linarias eran ya bien conocidas por los botánicos prelinneanos, que describieron numerosas especies (L'Écluse, 1576, 1601; Bauhin & Cherler, 1651; Boccone, 1674; Morison, 1680; Tournefort, 1700). Tournefort (1700) designó acertadamente *Linaria* como género independiente dentro de su grupo *de Herbis flore monopetalo, anomalo, tubulato, personato*. En cambio, Linneo (1753) incluyó las linarias en el género *Antirrhinum*, dentro de la clase *Didynamia* de su *Species Plantarum*. Poco después, Miller (1754) volvió a aceptar *Linaria* como un género separado, y proporcionó la primera

[1] Aunque no es frecuente encontrar el término castellanizado en la literatura botánica, la Real Academia Española lo recoge en su diccionario con el siguiente significado: "Planta herbácea de la familia de las Escrofulariáceas, con tallos erguidos ramosos, de cuatro a seis decímetros de altura, hojas parecidas a las del lino, estrechas, agudas, de color verde azulado y frecuentemente en verticilos, flores amarillas en espigas, y fruto capsular, ovoide, de dos celdas y muchas semillas menudas. Vive en terrenos áridos y se ha empleado en medicina como depurativo y purgante." La definición parece hacer referencia a alguna de las especies comunes de *Linaria* con flores amarillas, como *L. vulgaris* o *L. spartea*. Nosotros, sin embargo, hemos adoptado aquí el término para referirnos al conjunto del género, ya que nos ha parecido más apropiado que otros nombres comunes utilizados localmente para una o varias especies.

descripción postlinneana válida, con lo que el género quedó definitivamente reconocido como *Linaria* Mill.

En un principio, la circunscripción del género *Linaria* era más amplia que la actual, ya que incluía todas aquellas especies emparentadas con *Antirrhinum* que mostraban un espolón en la corola (Desfontaines, 1798; Lamarck & De Candolle, 1805; Chavannes, 1833; Bentham, 1846). Chavannes (1833) dividió el género en cuatro secciones: *Chaenorhinum*, *Cymbalaria*, *Elatinoides* and *Linariastrum*. Éstas fueron tratadas ya por Wettstein (1895) como géneros separados con los mismos nombres, a excepción de la sect. *Linariastrum*, que mantenía el nombre de *Linaria* (el género *Elatinoides* es conocido actualmente como *Kickxia*). Así, en *Linaria* quedaron incluidas aquellas especies con hojas enteras y sésiles, inflorescencias terminales racemosas (frente a las flores dispuestas en las axilas de las hojas de los otros tres géneros) y cápsulas con dehiscencia por medio de valvas. Ésta es, en esencia, la circunscripción de *Linaria* que se acepta actualmente, salvo por la discusión acerca de una serie de especies americanas, incluidas por algunos en *Linaria* (Pennell, 1935; Valdés, 1970b) y por otros en un género distinto, *Nuttallanthus* (Sutton, 1988) (véase abajo).

A lo largo del siglo XX y principios del XXI, se ha desarrollado un amplio abanico de estudios sobre el género *Linaria*, en los cuales se han utilizado las técnicas disponibles en cada momento para poner a prueba hipótesis sistemáticas, ecológicas y evolutivas. Hay que destacar los estudios de morfología y anatomía comparada (Champagnat, 1961; Valdés, 1968), micromorfología de polen y semillas (Viano, 1978d; Sutton, 1988; Juan *et al.*, 1999b), citogenética (Heitz, 1927; Valdés, 1969; Viano, 1971, 1973), biología reproductiva y de la polinización (Hill, 1909; Valdés, 1970c; Arnold, 1982; Docherty, 1982; Sánchez-Lafuente *et al.*, 2011), hibridación (East, 1933; Valdés, 1970d; Viano, 1978a), fitoquímica (Valdés, 1970a; Nikolova-Damyanova *et al.*, 1994; Beninger *et al.*, 2009) y paleobotánica (Allison *et al.*, 1952; Godwin, 1975; Szczepanek & Stachowicz-Rybka, 2004). Todo ello se ha acompañado de profundas revisiones sistemáticas y taxonómicas (Viano, 1969; Valdés, 1970b; Viano, 1978b, c; Sutton, 1988; Sáez & Crespo, 2005; Sáez & Bernal, 2009) y de la descripción de nuevas especies (Valdés & Cabezudo, 1977; Carretero & Boira, 1985; Crespo *et al.*, 1994) y táxones infragenéricos (Sutton, 1980). En las últimas décadas, la era del ADN y la biología molecular tampoco ha sido ajena al género *Linaria*, como muestran los estudios de genética del desarrollo (Hileman & Baum, 2003; Galego &

Almeida, 2007; Box *et al.*, 2011), epigenética (Cubas *et al.*, 1999), filogenia (Ghebrehiwet *et al.*, 2000; Vargas *et al.*, 2004) y genética de poblaciones (Segarra-Moragues & Mateu-Andrés, 2007; Ward *et al.*, 2008). Asimismo, con el despertar de la conciencia conservacionista también han proliferado los estudios sobre conservación de especies amenazadas (Benito *et al.*, 2009; Herreros *et al.*, 2009; Plaza & Rodríguez, 2009; Peñas *et al.*, 2011) y biología de la invasión (Peterson *et al.*, 2005; Sing & Peterson, 2011). En los siguientes epígrafes se revisarán algunos de los conocimientos disponibles sobre la biología del género *Linaria*, y se proporcionará así un marco para la investigación presentada en los subsiguientes capítulos de esta memoria. Se revisará con mayor profundidad la bibliografía disponible sobre la sección *Versicolores*, en la cual se centran tres de los cuatro capítulos principales de la memoria. Una especial atención se prestará, además, a *Linaria vulgaris*, la especie mejor estudiada del género. También se hará referencia a la información disponible sobre el género *Nuttallanthus*, potencialmente emparentado con *Linaria*.

Morfología

A continuación se incluye una descripción morfológica del género *Linaria*, basada en las de Sutton (1988) y Sáez & Bernal (2009):

> Hierbas anuales, bienales o perennes, pelosas o glabras; tallos heteromorfos o más o menos homomorfos, los tallos epicotilares normalmente escasos y degenerados, los tallos hipocotilares secundarios a menudo abundantes y dominantes, las ramas no cirrosas; tallos fértiles erectos, ascendentes o decumbentes, simples o ramificados, por lo general acompañados de tallos estériles decumbentes o ascendentes, simples. Hojas homomorfas o algo heteromorfas, simples, enteras, con nerviación pinnada o con varias venas principales subparalelas desde la base, sésiles, normalmente verticiladas en la parte inferior y alternas –en ocasiones opuestas– en la parte superior; hojas de los tallos estériles, cuando los hay, por lo general verticiladas. Inflorescencias en racimos bracteados terminales, espigas, panículas, o raramente flores solitarias en la axila de las hojas. Brácteas normalmente más pequeñas que las hojas del follaje, y que se reducen de tamaño de forma progresiva hacia el ápice, algunas veces parecidas a hojas, muy raramente axilando ejes adicionales. Flores zigomorfas, pentámeras, pediceladas o sésiles. Pedicelo por lo general acrescente en longitud, no cirroso, a veces recurvado o

reflejo. Cáliz profundamente dividido, con lóbulos enteros, valvados, iguales o desiguales, por lo general acrescentes; el lóbulo adaxial el más largo, o muy raramente el más corto, más corto o raramente más largo que el tubo de la corola. Corola personada con diversa coloración; limbo bilabiado, con los labios iguales o algo desiguales, el labio adaxial más o menos profundamente bilobulado, erecto-patente a patente, más corto o a veces más largo que el abaxial, el labio abaxial trilobulado, reflejo o patente, provisto de una convexidad bilobulada por lo general pelosa en la parte anterior (paladar), prominente, que ocluye total u ocasionalmente sólo parcialmente la boca del tubo (garganta); tubo más o menos cilíndrico, prolongado en la base en un espolón estrechamente cónico, linear o filiforme, recto o curvado, normalmente conspicuo; lóbulos enteros o muy raramente emarginados. Androceo didínamo, con 4 estambres fértiles, inclusos; filamentos con la base más o menos ensanchada; anteras con dehiscencia longitudinal, los pares adyacentes unidos marginalmente; conectivo no dilatado sobre las anteras; estaminodio diminuto. Gineceo bilocular; estilo más o menos recto, simple o bífido; estigma capitado, clavado, linear o a veces con 2 (-3) áreas estigmáticas discretas, situado entre pares de estambres fértiles. Fruto en cápsula bilocular, oblonga, ovoide o globosa, con paredes papiráceas, septo erecto, recto; lóculos con muchas semillas, iguales o desiguales –cuando son desiguales el adaxial excede al abaxial–, cada uno dehiscente por una apertura en poro solitaria por medio de 3-5 dientes o valvas desde el ápice hacia la base. Semillas dorsiventralmente o radialmente simétricas o algo asimétricas, ovoides, oblongas, reniformes, trígonas, tetraédricas o discoideas, crestadas, tuberculadas, alveoladas o lisas; hilo de subbasal a submedial, por lo general situado en la cresta marginal o el ala de las semillas discoideas, en algún caso en la cara plana o cóncava del disco; crestas de los ángulos o márgenes (1-) 2-6, delgadas y parecidas a alas, infladas o interrumpidas y escasamente evidentes; pared periclinal de las células de la testa tabular, tabular-convexa o raramente cóncava, lisa, verrucosa, rugulosa o reticulada, la de las células de los intersticios y lados de las crestas y tubérculos a menudo con papila medial o marginal, raramente bipapilosa; ceras epicuticulares normalmente ausentes.

En la Fig. 2 se muestra la forma típica de una flor personada de *Linaria* y se indican las partes de la corola. El desarrollo del paladar, una convexidad del labio inferior que cierra el acceso al interior de la corola en la mayoría de las especies de *Linaria*, es un carácter compartido con otros géneros de la tribu Antirrhineae, como *Antirrhinum*, *Cymbalaria*, *Kickxia*, *Misopates* y *Sairocarpus*. Además, la presencia en *Linaria* de un espolón portador de néctar en la base de la

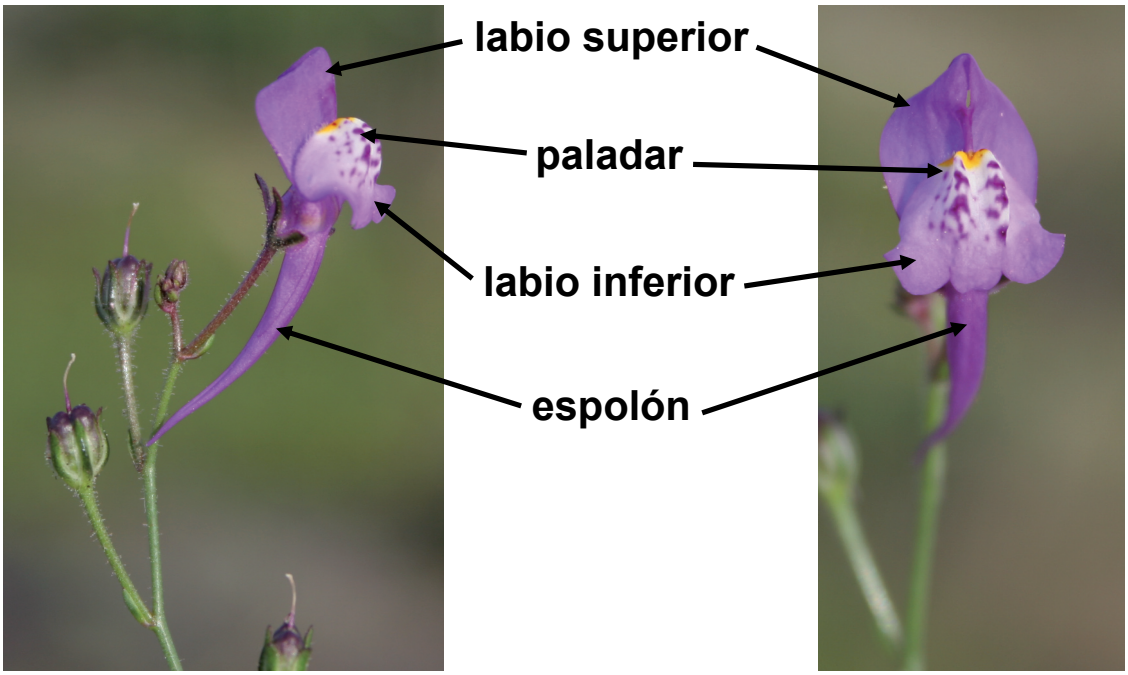


Fig. 2. Partes de la flor de *Linaria* en vista lateral (izquierda) y frontal (derecha). Se muestra *L. algarviana* como ejemplo. Fotografías de A. Fernández-Mazuecos.

corola implica una similitud con *Kickxia*, *Cymbalaria* y *Chaenorhinum*, géneros no estrechamente emparentados con *Linaria* (Ghebrehiwet *et al.*, 2000; Vargas *et al.*, 2004). El género que nos ocupa se diferencia de *Kickxia* por sus inflorescencias en racimo (frente a las flores solitarias y axilares de *Kickxia*), de *Cymbalaria* por sus hojas pinnatinervias (frente a las palmatinervias de *Cymbalaria*) y de *Chaenorhinum* por sus hojas sésiles (frente a las generalmente pecioladas de *Chaenorhinum*) (Sutton, 1988; Sáez & Bernal, 2009). Cuatro especies americanas se incluyeron anteriormente en *Linaria* (Pennell, 1935; Valdés, 1970b), pero fueron segregadas como un género independiente, *Nuttallanthus*, por Sutton (1988). Según este autor, *Linaria* y *Nuttallanthus* se diferencian por la forma del labio inferior de la corola (más grande en *Nuttallanthus*) y por caracteres de la semilla (cuatro a siete crestas longitudinales en *Nuttallanthus*, frente a un máximo de tres en *Linaria*, véase también la Tabla 2). En el Capítulo 2 se evaluará por primera vez la relación entre *Linaria* y *Nuttallanthus* mediante análisis filogenéticos.

Se han efectuado profundos análisis micromorfológicos de las semillas de *Linaria* mediante microscopía electrónica de barrido (Viano, 1979; Sutton, 1988; Juan *et al.*, 1999b; Segarra & Mateu, 2001a, b; Sáez *et al.*, 2004; Sáez & Crespo, 2005). Estos estudios han mostrado la

importancia, para la discriminación de secciones y especies, de los caracteres de la semilla, como la presencia o ausencia de un ala circundándola, la microescultura de la supeficie, la presencia de papila en las células de la testa, etc. Viano (1978d), por otra parte, realizó un estudio micromorfológico del polen de numerosas especies de semillas ápteras. Encontró un gran parecido entre especies, con granos de polen de estructura finamente reticulada (Fig. 3H). Cresti *et al.* (1988) describieron con gran detalle los caracteres morfológicos del grano de polen de *L. vulgaris*, de aspecto muy similar. También se ha estudiado la micromorfología de los pelos glandulares y no glandulares (Segarra & Mateu, 2001b; Sáez & Crespo, 2005) y de la superficie de las cápsulas (Juan *et al.*, 1999a).

Tratamientos taxonómicos

La clasificación infragenérica de *Linaria* ha seguido históricamente dos tendencias principales: algunos autores han dividido el género en dos grandes grupos dependiendo de la presencia o ausencia de un ala en las semillas (Morison, 1680; Dumortier, 1827; Boissier, 1879), mientras que otros lo han dividido en un número variable de secciones sobre la base de una gama más amplia de caracteres reproductivos y vegetativos (Chavannes, 1833; Bentham, 1846; Wettstein, 1895; Valdés, 1970b; Sutton, 1988). En otros casos se han combinado ambas aproximaciones (Viano, 1978b, c). Los estudios más recientes tienden a considerar que la clasificación basada en el ala de la semilla es artificial, dada la anatomía no homóloga de distintos tipos de ala que se pueden encontrar en el género (Valdés, 1970b; Sutton, 1988).

En los últimos tiempos se acepta de forma prácticamente universal la clasificación de Sutton (1988), que consta de 150 especies clasificadas en siete secciones y cinco subsecciones. En la Tabla 2 se resumen los principales caracteres que distinguen las secciones y subsecciones. Algunos autores añaden la sect. *Lectoplectron* (Pennell, 1935; Valdés, 1970b), equivalente al género *Nuttallanthus* de Sutton. Tanto el mismo Sutton como Valdés (1970b) ya propusieron la polifilia de la sección *Diffusae*, mientras que el resto de las secciones tienen caracteres mejor definidos que sugieren su monofilia. En cualquier caso, la condición de grupo natural de las secciones y subsecciones no se ha evaluado hasta el momento mediante técnicas filogenéticas. En la presente memoria se hace por primera vez utilizando tanto secuencias del ADN nuclear

Tabla 2. Clasificación infragenérica de *Linaria* según Sutton (1988). Se indican el número de especies reconocidas por el mismo autor, los caracteres morfológicos y la distribución de cada sección y subsección. Se incluye también el género *Nuttallanthus*, incluido por algunos autores en *Linaria* como sect. *Lectoplectron* (Valdés, 1970b).

Taxon	Nº de especies	Forma de la semilla	Ala de la semilla	Hábito	Lóbulo adaxial del cáliz	Estigma	Cáliz en fruto	Desarrollo del paladar	Distribución
Linaria Mill.									
Sect. *Linaria*	45	Discoidal, comprimida lateralmente	Con ala	Perenne	Largo	Entero	Pentámero	Prominente	Eurasia
Sect. *Speciosae* (Benth.) Wettst.	19	No discoidal	Sin ala	Perenne	Largo	Entero	Pentámero	Prominente	Región Mediterránea
Sect. *Diffusae* (Benth.) Wettst.	17	No discoidal	Sin ala	Anual y perenne	Largo	Entero	Pentámero	Prominente	Región Mediterránea
Sect. *Supinae* (Benth.) Wettst.	44								
Subsect. *Supinae*	32	Discoidal, comprimida lateralmente	Con ala o cresta marginal	Anual y perenne	Largo	Entero	Pentámero	Prominente	Región Mediterránea
Subsect. *Saxatile* Valdés	11	No discoidal	Generalmente con ala o cresta marginal	Anual y perenne	Largo	Entero	Pentámero	Prominente	Región Mediterránea
Subsect. *Trimerocalyx* (Murb.) D.A.Sutton	1	Discoidal, comprimida lateralmente	Con ala	Anual	Largo	Entero	Trímero	Prominente	Norte de África
Sect. *Pelisserianae* Valdés	2	Discoidal, comprimida dorsoventralmente	Con ala	Anual y perenne	Largo	Entero	Pentámero	Prominente	Región Mediterránea
Sect. *Versicolores* (Benth.) Wettst.	21								
Subsect. *Versicolores*	19	No discoidal	Sin ala	Anual y perenne	Largo	Dividido	Pentámero	Prominente o débil	Región Mediterránea
Subsect. *Elegantes* (Viano) D.A.Sutton	2	No discoidal	Sin ala	Anual	Largo	Emarginado	Pentámero	Débil	Península Ibérica
Sect. *Macrocentrum* D.A.Sutton	2	No discoidal	Sin ala	Anual	Corto	Entero	Pentámero	Débil	Región Mediterránea
Nuttallanthus D.A.Sutton (= sect. *Lectoplectron* Pennell)	4	No discoidal	Sin ala	Anual y bienal	Largo	Entero	Pentámero	Débil	América del Norte y América del Sur

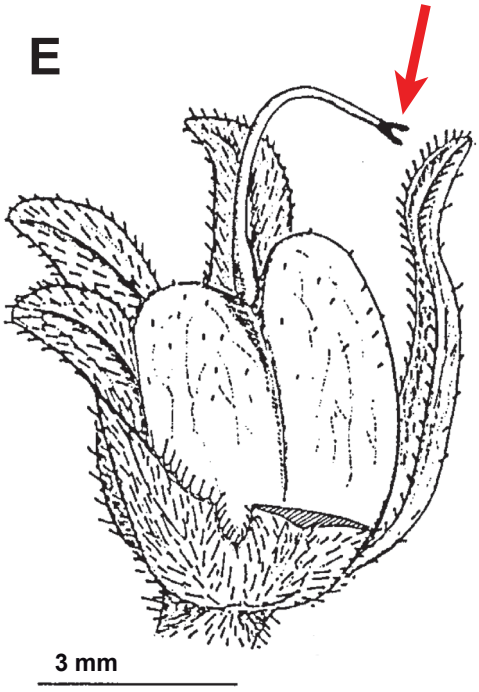
(Capítulo 2) como del ADN plastidial (Apéndice 2). Asimismo, se evalúa la condición de grupo natural de los dos grandes grupos con semillas aladas y no aladas.

La sección *Versicolores*, en su definición actualmente aceptada, es uno de los grupos de *Linaria* mejor definidos desde el punto de vista morfológico (Fig. 3; Sutton, 1988) y es el objeto de los Capítulos 3 a 5 y de los Apéndices 3 y 4 de la presente memoria. Originalmente, tuvo una circunscripción más amplia que la actual, ya que incluyó especies hoy incorporadas a las secciones *Macrocentrum* y *Speciosae*, así como al género *Nuttallanthus* (Bentham, 1846; Wettstein, 1895). De acuerdo con el tratamiento de Sutton (1988), la sect. *Versicolores* se distingue por su estigma bipartito, carácter exclusivo dentro del género e incluso de la tribu Antirrhineae (Fig. 3E). Este rasgo nos ha llevado a asignar al grupo el nombre común de "linarias bífidas" (*bifid toadflaxes* en inglés). Dentro de la sección, Sutton (1988) definió dos subsecciones: subsect. *Versicolores*, en la que el estilo está claramente dividido en dos lóbulos observables a simple vista, cada uno de ellos portador de un área estigmática; y la subsect. *Elegantes*, en la que los dos lóbulos son muy pequeños, de manera que el ápice del estigma es meramente emarginado. Aunque Viano (1978b, c) reconoció estos dos grupos como secciones independientes, los autores posteriores a Sutton generalmente han reconocido una sola sección con dos subsecciones (Sáez & Bernal, 2009). Las semillas de la sección *Versicolores* son ápteras (Fig. 3F, G) y presentan diversa ornamentación en la superficie (Fig. 3F), excepto en *L. pedunculata*, en la que son lisas (Fig. 3G).

La tipificación de la sect. *Versicolores* es problemática. La denominación asignada por Bentham (1846) sugiere que el tipo debería ser *L. versicolor* (Jacq.) Chaz. Sin embargo, este taxon,

Fig. 3. Caracteres morfológicos y citogenéticos de *Linaria* sect. *Versicolores*. (A-D) Diversidad floral: (A) *L. clementei*, (B) *L. pedunculata*, (C) *L. spartea*, (D) *L. elegans*. (E) Cápsula de *L. gharbensis*; la flecha indica el estigma bipartito típico de la subsect. *Versicolores*. (F-G) Semillas vistas al microscopio electrónico de barrido (MEB): (F) *L. incarnata*; (G) *L. pedunculata*. (H) Grano de polen de *L. elegans* al MEB. (I-J) Cromosomas en metáfase somática: (I) *L. multicaulis* subsp. *heterophylla*, 2n = 12; (J) *L. hellenica*, 2n = 24 (izquierda) y 2n = 26 (derecha). Fotografías de J. Ramírez (A, B), J. Quiles (C) y P. Vargas (D). E modificado de Juan *et al.* (1999a). F y G reproducidos de Juan *et al.* (1999b). H reproducido de Viano (1978d). I modificado de Viano (1971). J modificado de Contandriopoulos & Yannitsaros (1975).


A
B
C
D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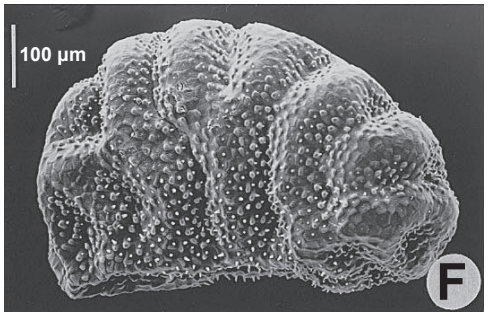
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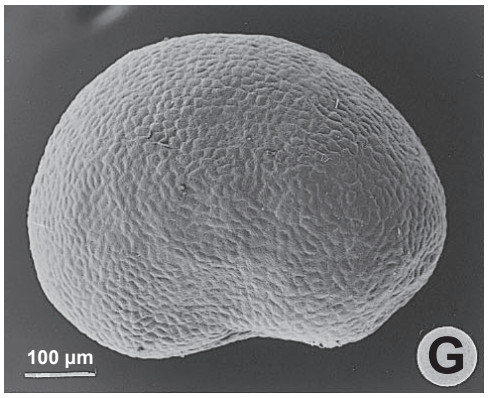
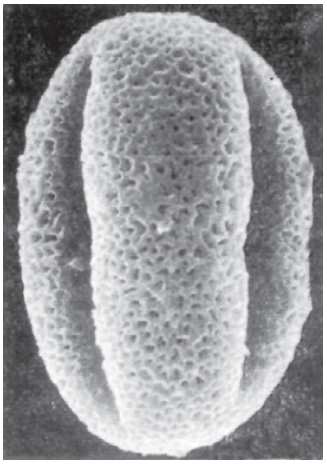
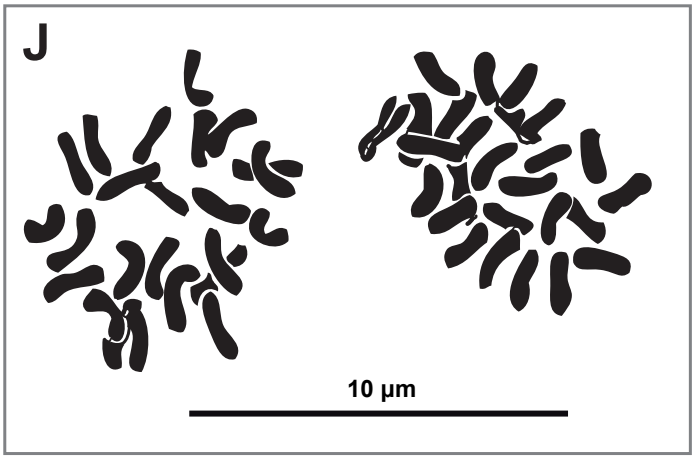
F


100 µm
G


H


I
10 µm


J
10 µm

supuestamente recolectado en Francia (Chavannes, 1833; Bentham, 1846), no aparece en los tratamientos taxonómicos modernos (Viano, 1978b, c; Sutton, 1988). Con toda probabilidad, tal nombre corresponde a plantas cultivadas de la sect. *Versicolores*, a veces designadas como *L. maroccana* hort., y seguramente obtenidas mediante hibridación entre distintas especies de origen marroquí (véase Usos) (Maire, 1941; Sutton, 1988). Debido a esta problemática, los autores recientes han optado por seleccionar un tipo diferente para la sección: *L. viscosa* (Viano, 1978b; Sutton, 1988).

El conjunto de caracteres empleado para distinguir las diferentes especies de la sect. *Versicolores* incluye el hábito (anual o perenne), la forma de los tallos, el tamaño y forma de las hojas, la densidad e indumento de las inflorescencias, la forma de las brácteas, la longitud de los pedicelos, la forma, tamaño e indumento de los sépalos, el tamaño y color de la corola (Figs. 3A-D), la longitud del espolón (Figs. 3A-D), la forma del estigma, la forma de las cápsulas y la ornamentación de las semillas (Figs. 3F, G), entre otros (Viano, 1978b, c; Sutton, 1988; Sáez & Bernal, 2009). Viano (1978b, c) reconoció 14 especies para su sect. *Versicolores* (= subsect. *Versicolores*) y dos para su sect. *Elegantes* (= subsect. *Elegantes*). Sutton (1988) amplió el número a un total de 21 especies (19 de la subsect. *Versicolores* y dos de la subsect. *Elegantes*) (Tabla 3). Mientras que Viano (1978b, c) reconoció un elevado número de táxones infraespecíficos con rango de subespecie, variedad y forma, Sutton (1988) únicamente reconoció subespecies para *L. multicaulis*, *L. viscosa* y *L. bordiana*. Autores posteriores han propuesto modificaciones al tratamiento de este autor. Así, para la polimorfa *L. multicaulis* se han descrito varias subespecies nuevas, a añadir a las cuatro reconocidas por Sutton, aunque apenas distinguibles de éstas (Dobignard, 1997; De Leonardis *et al.*, 1999; De Leonardis *et al.*, 2003). Además, se han descrito dos especies nuevas: *L. imzica* Gómiz, endémica del Antiatlas marroquí (Gómiz, 2004); y *L. iranica* Hamdi & Assadi, de Irán (Hamdi *et al.*, 2009), aunque ésta es probablemente asimilable a *L. tenuis*. Por otro lado, Tan & Iatrou (2001) han propuesto que *L. hellenica* es un sinónimo de *L. tenuis*, mientras que Fennane & Ibn Tattou (1998) han aceptado dos táxones marroquíes no reconocidos por Sutton: *L. gattefossei* Maire & Weiller y *L. viviesiae* Emb.

Tabla 3. Especies y subespecies de *Linaria* sect. *Versicolores* reconocidas por Sutton (1988). Para cada taxon se indican la distribución geográfica (Viano, 1978b, c; Sutton, 1988; Sáez & Bernal, 2009) y el número cromosómico (Valdés, 1969; Viano, 1971; Löve & Kjellqvist, 1974; Sutton, 1988; Díaz Lifante *et al.*, 1992). Éste es el tratamiento taxonómico que se ha sometido a test en la presente memoria doctoral.

Taxon	**Distribution**	**Número cromosómico**
Linaria sect. *Versicolores* (Benth.) Wettst.		
Subsect. *Versicolores*		
L. algarviana Chav.	SO de Portugal (Algarve)	2n = 12
L. bipartita (Vent.) Willd.	O de Marruecos	2n = 12
L. bordiana Santa & Simonneau		
subsp. *bordiana*	Argelia	Desconocido
subsp. *kralikiana* (Maire) D.A.Sutton	NO de África	Desconocido
L. clementei Haensel. ex Boiss.	S de España (Málaga)	2n = 12
L. dissita Pomel	NO de África	Desconocido
L. gharbensis Batt. & Pit.	NO de África, SO de España	2n = 12
L. hellenica Turrill	S de Grecia	2n = 24, 26
L. incarnata (Vent.) Spreng.	O de la península Ibérica	2n = 12
L. maroccana Hook.f.	Marruecos (principalmente Alto Atlas)	2n = 12
L. multicaulis (L.) Mill.		
subsp. *multicaulis*	Sicilia, S de Italia (Calabria)	2n = 12
subsp. *aurasiaca* (Pomel) D.A.Sutton	Túnez, NE de Argelia	Desconocido
subsp. *galioides* (Ball) D.A.Sutton	Marruecos (Alto Atlas)	2n = 12
subsp. *heterophylla* (Desf.) D.A.Sutton	NO de África	2n = 12
L. pedunculata (L.) Chaz.	S de la península Ibérica, NO de África, Islas Baleares	2n = 12
L. pinifolia (Poir.) Thell.	Túnez, Argelia	2n = 12
L. pseudoviscosa Murb.	Túnez	2n = 12
L. salzmannii Boiss.	S de España (Málaga)	2n = 12
L. spartea (L.) Chaz.	Península Ibérica, S de Francia	2n = 12
L. tenuis (Viv.) Spreng.	N de África, Oriente Medio	2n = 12
L. tingitana Boiss. & Reut.	NO de África	2n = 12
L. viscosa (L.) Chaz.		
subsp. *viscosa*	S de la península Ibérica	2n = 12
subsp. *spicata* (Coutinho) D.A.Sutton	SE de la península Ibérica	2n = 12
subsp. *crassifolia* (Coutinho) D.A.Sutton	CO de Portugal	Desconocido
L. weilleri Emb. & Maire	S de Marruecos (Anti Atlas)	
Subsect. Elegantes (Viano) D.A.Sutton		
L. elegans Cav.	NO de la península Ibérica	2n = 12
L. nigricans Lange	SE de España (Almería)	Desconocido

Citogenética

Linaria parece ser un género bastante homogéneo y estable desde el punto de vista citogenético. El número cromosómico básico es x = 6, al igual que en *Nuttallanthus* (Heitz, 1926, 1927; Valdés, 1969; Viano, 1971; Sutton, 1988). Por lo general, los cromosomas son metacéntricos o submetacéntricos y de pequeño tamaño (Valdés, 1969; Viano, 1971). Las especies de las secciones *Linaria*, *Pelisserianae* y *Speciosae* parecen tener cromosomas más grandes que los de las secciones *Versicolores* y *Supinae* (Viano, 1971; Fernandes *et al.*, 1977; Sutton, 1988).

La gran mayoría de las especies de *Linaria* para las cuales se han realizado recuentos cromosómicos son diploides (2n = 12, revisado por Sutton, 1988). La poliploidía parece ser extremadamente rara. Sólo se conocen tres especies tetraploides (2n = 24): *L. chalepensis*, de la sect. *Macrocentrum* (Heitz, 1927); *L. pelisseriana*, de la sect. *Pelisserianae* (Larsen & Laegaard, 1971); y *L. hellenica*, de la sect. *Versicolores* (Contandriopoulos & Yannitsaros, 1975). También es tetraploide *Nuttallanthus texanus* (Lewis, 1958). Además, al menos una especie de *Linaria* (*L. angustissima*) es polimórfica, con individuos diploides y tetraploides (2n = 12, 24) (véase Sutton, 1988). En ninguno de los casos anteriores se conoce si los tetraploides son de origen autopoliploide o alopoliploide.

En el caso de la sect. *Versicolores*, se conoce el número cromosómico para la mayoría de los táxones (Tabla 3). Todas las especies estudiadas son diploides (2n = 12; Fig. 3I) excepto *L. hellenica*, que es tetraploide (Fig. 3J). En ésta, no sólo se han encontrado individuos con número cromosómico 2n = 24, sino también individuos tetrasómicos con 2n = 26 (Fig. 3J), lo que indica un evento de aneuploidía (Contandriopoulos & Yannitsaros, 1975). Por otro lado, en algunos individuos de *L. bipartita* se ha detectado la presencia de un cromosoma B o cromosoma supernumerario (Nazeer *et al.*, 1980).

Aunque aún faltan recuentos cromosómicos de un elevado número de especies de *Linaria* (en particular de muchas especies asiáticas poco conocidas de la sect. *Linaria*), los datos disponibles indican que los cambios en el número de cromosomas no han tenido una gran importancia en la historia evolutiva del género (Viano, 1971). Se ha sugerido que las escasas especies poliploides son probablemente el resultado de eventos recientes (Sutton, 1988), aunque esta hipótesis no se ha comprobado mediante herramientas filogenéticas.

Biología celular

Vale la pena mencionar las inusuales inclusiones cristaloides que se han encontrado en el interior del núcleo de células de las hojas y estilos de *L. vulgaris* (Ciampolini *et al.*, 1980). Están formadas por paquetes de túbulos ordenados regularmente, con aspecto similar a los microtúbulos del citoplasma. La función de estas estructuras es desconocida, aunque parecen estar relacionadas con el nucleolo.

Biología reproductiva

Aunque se ha descrito la presencia de reproducción vegetativa mediante yemas adventicias radicales, estolones y tallos rizomatosos subterráneos (p.ej. en *L. vulgaris* y *L. repens*; Bakshi & Coupland, 1960; Sutton, 1988), las linarias se reproducen principalmente de forma sexual. Las flores son casmógamas, es decir, se desarrollan completamente, de manera que los órganos masculinos y femeninos quedan potencialmente accesibles para los agentes polinizadores. En las especies americanas de *Nuttallanthus*, sin embargo, también se ha descrito la presencia de flores cleistógamas, flores diminutas que no llegan a abrirse y que contribuyen, por autopolinización, a alargar el periodo de fructificación (Hill, 1909; Crawford & Elisens, 2006). En *Linaria*, se apuntó hace más de un siglo la existencia de flores cleistógamas hipogeas (que se desarrollan y fructifican bajo la tierra) al menos en un taxon, *L. reflexa* subsp. *agglutinans* (Hill, 1909), aunque este interesante caso no se ha confirmado desde entonces.

Los estudios de biología reproductiva publicados sugieren una predominancia de la alogamia (fecundación cruzada) en el género, como muestra el hecho de que la mayor parte de las especies estudiadas son autoincompatibles (Bruun, 1937; Valdés, 1970c; Docherty, 1982). Se ha postulado para el género un sistema de autoincompatibilidad gametofítica controlado por un locus (Docherty, 1982). Con todo, se ha encontrado un mayor o menor grado de autocompatibilidad en varias especies pertenecientes a distintas secciones de *Linaria*, así como en *Nuttallanthus* (Bruun, 1937; Valdés, 1970c; Docherty, 1982; Crawford & Elisens, 2006). En *Nuttallanthus*, se ha encontrado un sistema reproductivo predominantemente autógamo asociado a la producción

de flores cleistógamas, como se ha comentado arriba (Hill, 1909; Crawford & Elisens, 2006). De manera análoga, un grupo de especies autógamas de la sect. *Supinae* muestran flores diminutas que, sin llegar a ser cleistógamas, apenas resultan atractivas para los polinizadores (Hill, 1909; Valdés, 1970c; Valdés & Díaz-Lifante, 1996; Segarra-Moragues & Mateu-Andrés, 2007). En la sect. *Versicolores*, parecen ser esencialmente autoincompatibles *L. bipartita*, *L. incarnata*, *L. multicaulis*, *L. spartea* y *L. viscosa*. *Linaria pedunculata*, por el contrario, es autocompatible, mientras que en *L. maroccana* y *L. pinifolia* se han obtenido resultados dispares (Bruun, 1937; Docherty, 1982). Por otro lado, las flores de *Linaria* son protóginas, ya que el estigma es receptivo antes de que maduren las anteras (Valdés, 1970c).

Polinización, dispersión y otras interacciones

La elaborada forma de las flores de *Linaria* es un claro indicador de adaptación a la polinización entomófila. Los polinizadores son insectos que pueden ser atraídos por los atractivos colores (amarillo, púrpura, rojizo) de las corolas. En muchas especies, el contraste entre el color del paladar y el del resto de la corola puede tener un importante papel en esta atracción (Valdés, 1970c; Kampny, 1995). El olor tendría menos importancia, excepto en algunos casos como *L. odora*, *L. reflexa* subsp. *agglutinans* y *L. aeruginea* subsp. *pruinosa* (Sutton, 1988). En las especies de corola ocluida (personada), que son mayoría, el polinizador debe ser capaz de separar el paladar del labio superior, para acceder de este modo al interior de la corola, donde las guías nectaríferas generalmente presentes en la parte ventral del tubo lo dirigen hacia la recompensa en forma de néctar contenida en el espolón (Valdés, 1970c). El néctar está compuesto, al menos en *L. vulgaris*, principalmente por sacarosa y cantidades menores de otros azúcares, como glucosa, fructosa y rafinosa (Arnold, 1982; Nepi *et al.*, 2003). En el proceso de acceder al néctar, el polinizador carga polen generalmente en el *scutum* (parte dorsal del tórax) por contacto con las anteras, y lo transfiere al estigma (situado entre los dos pares de anteras) de las flores que visita a continuación (Sprengel, 1793; Newman & Thomson, 2005a). Ésta es la forma más típica de polinización en *Linaria*, de tipo nototríbico (Macior, 1967; Kampny, 1995).

Desde hace tiempo se ha considerado que la flor de *Linaria* es, al igual que la de *Antirrhinum*, un ejemplo típico de melitofilia, es decir, adaptación a la polinización por abejas (Robertson,

1888). Aunque hay pocos estudios de visitantes florales, en los disponibles las abejas de géneros como *Bombus*, *Apis* y *Anthophora* (Apidae, abejas de tamaño generalmente grande y probóscide relativamente larga) parecen ser los principales polinizadores. Es el caso de *L. vulgaris* (Arnold, 1982; Stout *et al.*, 2000; Newman & Thomson, 2005a) y *L. lilacina* (Sánchez-Lafuente, 2007; Sánchez-Lafuente *et al.*, 2011), especies de flores robustas con un paladar bien desarrollado que probablemente restringe el acceso de lepidópteros y dípteros. Abejas más pequeñas (p.ej. del género *Halictus*, Halictidae) también acceden ocasionalmente a las flores tanto de *L. vulgaris* como de *L. lilacina*, aunque su efectividad como polinizadores parece ser menor (Arnold, 1982; Sánchez-Lafuente *et al.*, 2011). La polinización por lepidópteros parece ser más rara, aunque sí se han registrado en *L. vulgaris* visitas por parte de *Macroglossum stellatarum* (Sphingidae) y de algunas mariposas (Knoll, 1922; Nepi *et al.*, 2003). Esto podría ser relativamente frecuente en ciertas especies con flores más pequeñas y probablemente más fáciles de abrir, por ejemplo *L. alpina* (Müller, 1881). Se ha propuesto, incluso, que las flores abiertas de paladar poco desarrollado y tubo estrecho de la sect. *Macrocentrum* y de *Nuttallanthus* podrían favorecer la polinización por lepidópteros (Robertson, 1888; Hill, 1909; Pennell, 1935; Sutton, 1980, 1988), y que la flor de *N. floridanus*, prácticamente carente de espolón, podría ser polinizada por dípteros (Pennell, 1935). La sect. *Versicolores* proporciona un interesante grupo de estudio para poner a prueba hipótesis de evolución morfológica asociada a cambios de polinizadores, ya que en ella se incluyen especies morfológicamente dispares, algunas con el paladar bien desarrollado y otras con un labio inferior que no llega a cerrar el acceso a la garganta, además de una amplia diversidad de longitudes del espolón y anchuras del tubo floral (Viano, 1969, 1978b, c). A ello se dedica el Capítulo 4 de esta memoria.

Apenas se han estudiado los procesos de dispersión de semillas en *Linaria*, pese a que la presencia en el género de dos tipos dispares de semillas (aladas y ápteras) constituye un interesante caso para un análisis comparativo (Viano, 1978c; Sutton, 1988). Se ha sugerido que el pequeño tamaño de las semillas y la presencia de alas, crestas y tubérculos están asociados a la dispersión por viento o anemocoria (Sutton, 1988). Sin embargo, en *L. vulgaris*, una especie de semillas aladas, se ha encontrado que una gran parte de las semillas (> 80%) cae a menos de 0,5 m de la planta madre, y muy pocas a más de 1,5 m (Nadeau & King, 1991). Por el momento, no se ha evaluado hasta qué punto son frecuentes los fenómenos de dispersión a larga distancia (véase el Capítulo 2 de esta memoria). En *L. japonica*, se ha sugerido una adaptación a la dispersión

por el mar debido a la presencia de un ala muy desarrollada, gruesa, y con textura de corcho (Sutton, 1988). También se han descrito casos puntuales de endozoocoria (mediante pájaros, en *L. vulgaris*) y mirmecocoria (por hormigas, en *L. simplex*), aunque parece improbable que estos fenómenos sean importantes en la dispersión de *Linaria* (Sutton, 1988).

Además de la polinización (y casos puntuales de dispersión), las linarias se ven involucradas en otras interacciones con animales, en especial con insectos. Varios estudios han evaluado el efecto que tienen el robo de néctar (por parte de abejas de géneros como *Bombus* y *Xylocopa*, que pican el espolón sin contactar con los órganos reproductivos) y la depredación de semillas sobre el éxito reproductivo de *L. vulgaris* (Arnold, 1982; Stout *et al.*, 2000; Nepi *et al.*, 2003; Newman & Thomson, 2005a, b). Se ha encontrado que la depredación de semillas tiene un efecto negativo importante (véase abajo), mientras que el efecto del robo de néctar es reducido (Arnold, 1982; Stout *et al.*, 2000). De hecho, se ha encontrado que el robo de néctar podría incluso tener un efecto positivo (Newman & Thomson, 2005b). En cualquier caso, dado que la mayoría de los estudios mencionados se han realizado fuera del área natural de *L. vulgaris* (excepto el de Stout *et al.*, 2000), hay que ser cauto a la hora de extrapolar sus conclusiones a poblaciones autóctonas. En poblaciones naturales de *L. lilacina* sí se ha descrito recientemente un efecto negativo de la herbivoría de las corolas sobre la atracción de los polinizadores, y con ello, sobre el éxito reproductivo (Sánchez-Lafuente, 2007).

Mención aparte merecen las interacciones de las linarias con distintas especies de escarabajos (Coleoptera) que frecuentemente se encuentran sobre las plantas y que suelen afectar negativamente a su éxito reproductivo (McClay, 1992; Wilson *et al.*, 2005). Los más frecuentes son *Brachypterolus pulicarius* (Kateretidae) (Newman & Thomson, 2005b) y distintos gorgojos (Curculionidae), como *Mecinus janthinus* (Jeanneret & Schroeder, 1992; Wilson *et al.*, 2005) y varias especies del género *Rhinusa* (*R. antirrhini*, *R. neta*, *R. linariae*, anteriormente incluidas en *Gymnaetron*) (Wilson *et al.*, 2005; Caldara *et al.*, 2010). *Rhinusa* es un género de gran interés para el estudio de procesos de coevolución planta-insecto, ya que las distintas especies presentan una cierta especificidad en la selección de las plantas hospedadoras (Caldara *et al.*, 2010). En un reciente análisis filogeográfico de *R. antirrhini* se ha encontrado una marcada divergencia de linajes asociados de manera específica a distintas especies hospedadoras de *Linaria* (Hernández-Vera *et al.*, 2010).

Hibridación

Desde antiguo se conoce la existencia de híbridos interespecíficos de *Linaria* en la naturaleza (Brébisson, 1859). Son especialmente comunes los híbridos entre especies de la misma sección. También parecen ser frecuentes los híbridos entre especies de las secciones *Linaria* y *Speciosae* (pese a tratarse de grupos con semillas aladas y no aladas respectivamente), y de éstas con especies de la sect. *Supinae* (revisado por Valdés, 1970d; Viano, 1978a). Dentro de la sect. *Versicolores*, se han descrito varios híbridos espontáneos, enumerados en la Tabla 4.

Tabla 4. Híbridos naturales y experimentales de *Linaria* sect. *Versicolores* registrados en la bibliografía. Para los híbridos naturales, el orden de los parentales no refleja necesariamente cuál de ellos actúa como progenitor masculino o femenino. Para los híbridos experimentales, el primer parental es el que actúa como progenitor femenino, y el segundo como masculino.

Híbrido	**Referencia**
Híbridos naturales	
L. gharbensis x L. bipartita (= *L. x zaborskiana* Emb.)	Emberger (1930)
L. gharbensis x L. maroccana (= *L. x polychroa* Gattef. & Maire)	Maire (1941)
L. algarviana x L. incarnata	Viano (1973)
L. gharbensis x L. viscosa subsp. *viscosa*	Sáez & Bernal (2009)
L. spartea x L. viscosa subsp. *viscosa*	Sáez & Bernal (2009)
Híbridos experimentales fértiles	
L. algarviana x L. gharbensis	Viano (1978a)
L. gharbensis x L. algarviana	Viano (1978a)
L. bipartita x L. gharbensis	Viano (1978a)
L. gharbensis x L. bipartita	Viano (1978a)
L. bipartita x L. maroccana	Viano (1978a)
L. gharbensis x L. maroccana	Viano (1978a)
L. maroccana x L. gharbensis	Viano (1978a)
L. spartea x L. pseudoviscosa	Viano (1978a)
L. viscosa x L. pseudoviscosa	Viano (1978a)
L. spartea x L. gharbensis	Viano (1978a)
Híbridos experimentales parcial o totalmente estériles	
L. bipartita x L. algarviana	Viano (1978a)
L. algarviana x L. bipartita	Viano (1978a)
L. multicaulis subsp. *heterophylla x L. pseudoviscosa*	Viano (1978a)
L. pseudoviscosa x L. multicaulis subsp. *heterophylla*	Viano (1978a)
L. algarviana x L. pseudoviscosa	Viano (1978a)
L. pseudoviscosa x L. algarviana	Viano (1978a)
L. maroccana x L. bipartita	Viano (1978a)
L. viscosa x L. bipartita	Valdés (1970d)
L. bipartita x L. viscosa	Valdés (1970d)

A lo largo del siglo XX, varios estudios evaluaron en detalle las barreras reproductivas entre especies de *Linaria* mediante estudios de hibridación interespecífica experimental (East, 1933; Bruun, 1937; Valdés, 1970d; Viano, 1978a). Se ha confirmado que las especies de las secciones *Linaria*, *Speciosae* y *Supinae* hibridan con relativa facilidad, tanto dentro de cada sección como entre las tres secciones, aunque los híbridos parecen ser casi siempre estériles (Valdés, 1970d). Por el contrario, las especies de la sect. *Versicolores* no hibridan con especies de otras secciones (Valdés, 1970d; Viano, 1978a). En la sect. *Versicolores*, las especies de la subsect. *Elegantes* no hibridan con las de la subsect. *Versicolores*, mientras que dentro de la subsect. *Versicolores* se obtienen híbridos con cierta facilidad (Tabla 4; Valdés, 1970d; Viano, 1978a). Buena parte de estos híbridos son fértiles, aunque es frecuente la presencia de anomalías meióticas (East, 1933; Viano, 1978a). En general, los híbridos presentan caracteres morfológicos intermedios respecto a los de los parentales (Viano, 1978a).

Se ha interpretado que la debilidad de las barreras reproductivas interespecíficas dentro de las secciones de *Linaria* es evidencia de una especiación reciente y probablemente asociada a barreras geográficas (especiación alopátrida) (Viano, 1978a). Asimismo, la hibridación en la naturaleza se vería facilitada por la ausencia de una especificidad planta-polinizador marcada en la mayoría de los casos (Valdés, 1970d).

Hábitat

Las linarias ocupan una amplia variedad de hábitats, especialmente en la región Mediterránea (Sutton, 1988; Sáez & Bernal, 2009). El rango altitudinal del género va desde el nivel del mar (*L. polygalifolia*, *L. pedunculata*) hasta altitudes superiores a los 3000 m (*L. alpina*, *L. glacialis*). La mayor parte de las especies de las secciones *Supinae*, *Speciosae*, *Versicolores*, *Diffusae*, *Macrocentrum* y *Pelisserianae* están adaptadas a un clima mediterráneo o submediterráneo (en sus diversas variantes), mientras que en la sect. *Linaria* se pueden encontrar especies más propias de climas eurosiberianos (*L. vulgaris*) o continentales extremos (*L. baligaliensis*). Aunque *L. elegans* y *L. spartea* llegan a aparecer en zonas de clima atlántico, las especies de la sect. *Versicolores* son típicamente mediterráneas, y pueden vivir en arenales costeros (*L. pedunculata*), zonas abiertas y bordes de cultivos (*L. spartea*, *L. bipartita*, *L. gharbensis*),

dehesas y pastos (*L. incarnata*, *L. spartea*), áreas subdesérticas (*L. nigricans*, *L. tenuis*), claros de bosques (*L. elegans*), claros de matorral (*L. algarviana*), medias y altas montañas (*L. elegans*, *L. maroccana*, *L. multicaulis* subsp. *galioides*), etc. En general, las especies de *Versicolores* prefieren sustratos arenosos, bien de naturaleza ácida (*L. spartea*, *L. incarnata*) o básica (*L. clementei*, *L. salzmannii*). A veces aparecen también en roquedos (*L. viscosa* subsp. *spicata*).

Fitoquímica

Se ha aislado un buen número de compuestos químicos del metabolismo secundario de especies de *Linaria*. Como en otros géneros de Antirrhineae, es característica la presencia de glucósidos iridoides, como el antirrhinósido y el linariósido (Marco, 1985; Ilieva *et al.*, 1992; Handjieva *et al.*, 1993; Nikolova-Damyanova *et al.*, 1994). Dentro de la sect. *Versicolores*, en *L. clementei* se han aislado cuatro glucósidos iridoides diferentes, dos de ellos aparentemente nuevos para la ciencia (Marco, 1985). Las plantas utilizan este grupo de compuestos monoterpenoides como defensa frente a microorganismos y frente a herbívoros generalistas, al tiempo que pueden atraer y estimular a ciertos especialistas adaptados a ellos (Jamieson & Bowers, 2010).

Valdés (1970a) y Gibbs (1974) analizaron los pigmentos florales (flavonoides) de 17 especies de *Linaria* pertenecientes a cinco secciones diferentes, incluidas cinco especies de la sect. *Versicolores*. Valdés encontró un total de seis auronas, diecisiete flavonas, dos flavonoles y dos antocianinas diferentes en el género. De acuerdo con estos resultados, los colores amarillos de las flores estarían determinados por la presencia, en las vacuolas de las células de los pétalos, de auronas como el aureusidín-6-glucósido y el bracteatín-6-glucósido. Los colores violetas, púrpuras, marrones y crema estarían determinados por la presencia de antocianinas: cianidín-3-glucósido, cianidín-3-rutinósido, cianidín-3,5-diglucósido y delfinidín-3,5-diglucósido. La acción de los pigmentos se vería modificada por el pH, así como por otros flavonoides y ácidos cinámicos (Valdés, 1970a). Valdés (1970a) también estudió los flavonoides presentes en las hojas, y mostró su potencial valor taxonómico (Valdés, 1970a; Harborne & Valdés, 1971).

Desarrollo, genética y epigenética

El desarrollo vegetativo y reproductivo de *Linaria* ha suscitado un buen número de publicaciones. Cook (1924) describió el desarrollo del embrión y la semilla. Champagnat (1952, 1958, 1961) efectuó detallados estudios morfológicos y anatómicos del desarrollo de los tallos, y describió interesantes diferencias entre especies perteneciente a diferentes secciones (véase también Sutton, 1988). Una plántula de *Linaria* desarrolla inicialmente un tallo epicotilar (por encima de los cotiledones) apical. En la sect. *Versicolores*, entre otras, este eje primario se marchita pronto, y se desarrollan ejes secundarios hipocotilares (originados por debajo de los cotiledones), que son más robustos y darán lugar a tallos fértiles. Es frecuente la presencia de tallos hipocotilares dimórficos, es decir, tallos estériles y fértiles de diferente tipo (frecuentemente con hojas verticiladas en los tallos estériles y alternas en los fértiles) (Sutton, 1988).

El desarrollo de la compleja flor de *Linaria* ha intrigado a los científicos desde antiguo. La existencia de mutantes de *L. vulgaris* con una flor más o menos actinomorfa ya fue puesta de manifiesto por Linneo (1749). Asignó estas plantas a un género distinto, *Peloria* (del griego *pelōr*, que significa "monstruo"), y admitió que éste se debía haber originado a partir de *Linaria* por medio de una transformación o mutación, lo que suponía un desafío para su propia tesis acerca de la constancia de las especies (Gustafsson, 1979). El término "flor pelórica" se ha aplicado desde entonces a todos los mutantes de flor más o menos actinomorfa que, con cierta frecuencia, aparecen en especies de flor zigomorfa de diversas familias. El problema de la peloria interesó posteriormente a Goethe (1820), Darwin (1868) y De Vries (1901-1903). La peloria se ha encontrado en numerosas especies de *Linaria* (Fig. 4A, B) y otras antirrhineas, y puede ser completa o parcial (Masters, 1869; Camp & Gilly, 1941; Sutton, 1987). Camp & Gilly (1941) describieron otros casos de anomalías florales en *L. vulgaris*, incluidas flores sin espolón y flores tetrámeras.

El avance de las técnicas de la genética molecular y de la biología del desarrollo en los últimos años ha permitido analizar en detalle el desarrollo de la flor de *Linaria* (Fig. 4C-L) y la base molecular de la peloria. Hoy se sabe que dos genes parálogos (procedentes de un evento de duplicación) denominados *CYCLOIDEA* (*CYC*) y *DICHOTOMA* (*DICH*) controlan, entre otros, la zigomorfía en las antirrhineas (Gübitz *et al.*, 2003; Hileman & Baum, 2003; Hileman *et al.*, 2003). Cubas *et al.* (1999) estudiaron la función del ortólogo de *CYC* en *L. vulgaris*, denominado *Lcyc*.

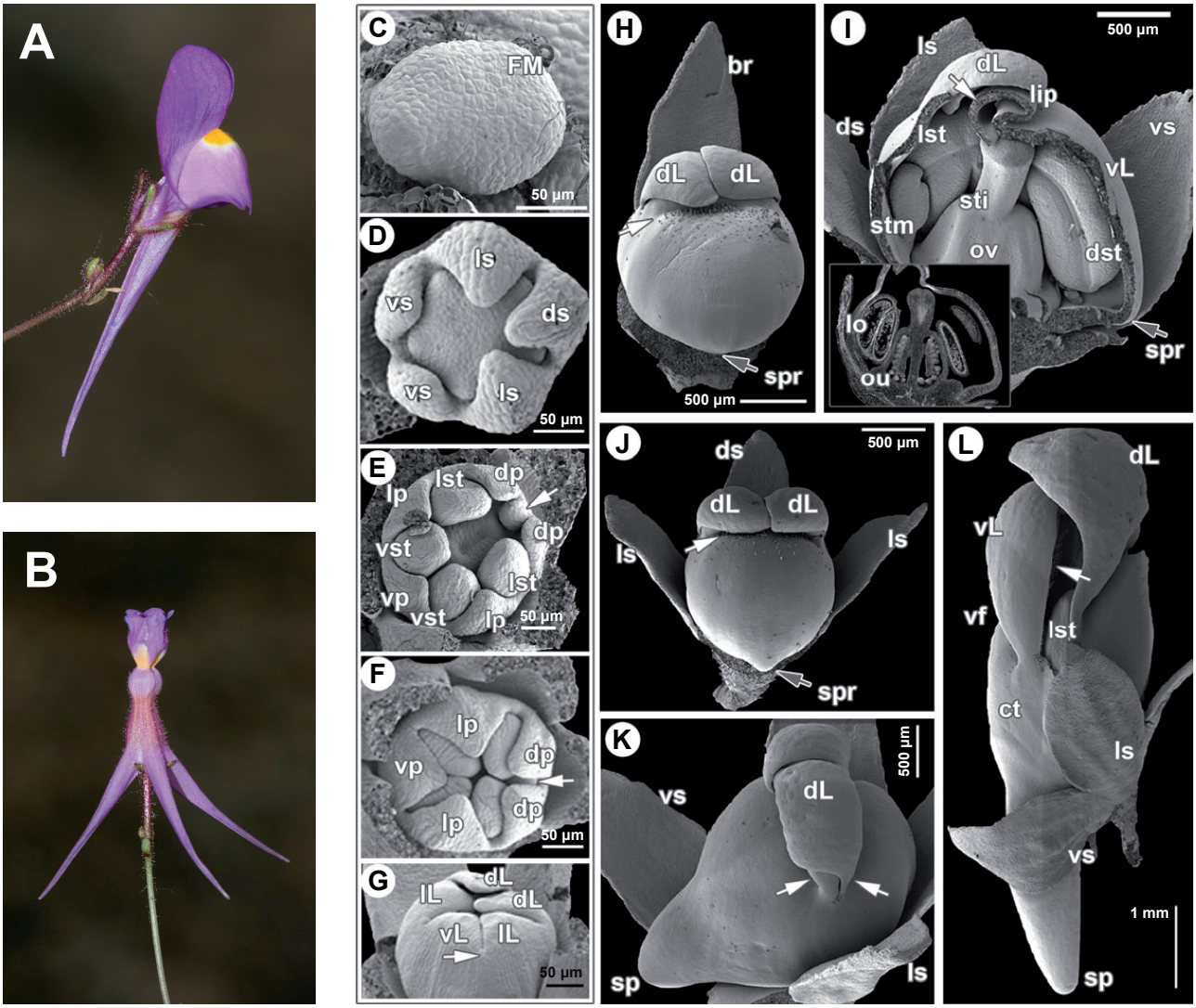


Fig. 4. (A, B) Flor normal (A) y pelórica (B) de *L. salzmannii*. (C-L) Ontogenia de la flor de *L. vulgaris*. Abreviaturas: ct, tubo de la corola; dL, lL y vL, lóbulos de los pétalos dorsal, lateral y ventral; dp, lp y vp, pétalos; ds, ls and vs, sépalos; lo, lóculo de antera; ov, ovario; ou, óvulo; sp, espolón; spr, primordio del espolón; sti, estigma; stm, estaminodio; vf, surco ventral; vst y lst, estambres ventral y lateral. Fotografías A y B de J.L. Blanco-Pastor. C-L reproducido de Box *et al.* (2011).

Demostraron que la peloria en esta especie es causada por el silenciamiento de *Lcyc* debido a una modificación epigenética heredable del gen. En concreto, el ADN de *Lcyc* está fuertemente metilado en los individuos pelóricos, pero no en los de flor zigomorfa.

Recientemente se ha abordado también el desarrollo del espolón de *Linaria* desde los puntos de vista morfológico y genético (Box *et al.*, 2011). El espolón de *L. vulgaris* (que nuevamente se ha utilizado como modelo) deriva del pétalo ventral (Fig. 4C-L), y su elongación se asocia a la

expansión longitudinal de las células. Se han aislado dos genes homeóticos de la familia *KNOX*, designados como *LvHirz* y *LvIna*, que estarían implicados en el desarrollo del espolón (Box *et al.*, 2011). Cambios en la regulación de estos genes podrían estar involucrados en la aparición del espolón en *Linaria* y otros géneros de Antirrhineae.

La base genética y ontogenética de la variación del color de la corola es bien conocida en *Antirrhinum*, donde los locus *ROSEA* (*ROS*), *ELUTA* (*EL*) y *SULFUREA* (*SULF*) regulan la síntesis de antocianinas y auronas (Schwinn *et al.*, 2006; Whibley *et al.*, 2006). Presumiblemente, en *Linaria* actuarían mecanismos similares, pero apenas se han investigado. Únicamente se ha estudiado la base molecular de la variegación de la corola en ciertas variedades cultivadas de *Linaria* sect. *Versicolores* (Galego & Almeida, 2007), que sería debida a la inestable inserción de un transposón en el gen *Lpal*, que codifica para la dihidroflavonol-4-reductasa, una enzima requerida para la síntesis de antocianinas.

Biogeografía

Linaria es un género paleártico con su centro de diversidad en la región Mediterránea (Sutton, 1988), de la que seis de las siete secciones son endémicas o subendémicas (Fig. 5). La única excepción es la sect. *Linaria*, que está escasamente representada en esta región. Por el contrario, tiene su centro de diversidad en el suroeste de Asia y extiende su distribución por buena parte de Eurasia hasta el Extremo Oriente (*L. japonica*). Las seis secciones mediterráneas se pueden considerar, en un sentido amplio, como circunmediterráneas, pero la diversidad está desigualmente repartida dependiendo de la sección (Sutton, 1988).

El género *Nuttallanthus* es exclusivo del Nuevo Mundo (Fig. 5), con tres especies en Norteamérica y una (*N. subandinus*) en Suramérica (Diels, 1906; Pennell, 1935; Sutton, 1988). Se ha debatido acerca del parentesco entre las especies del Viejo Mundo de *Linaria* y las americanas de *Nuttallanthus*. Algunos autores han propuesto una estrecha relación entre ellas (Pennell, 1935; Valdés, 1970b). Se ha sugerido que las especies americanas se originaron por migración desde la región Paleártica, dada la mayor diversidad en ésta (Pennell, 1935; Hong, 1983). Sin embargo, se desconoce cuándo ocurrió esta conexión, y si se produjo a través de la zona del estrecho de Bering o del Atlántico Norte (Pennell, 1935; Hong, 1983). La aplicación de técnicas

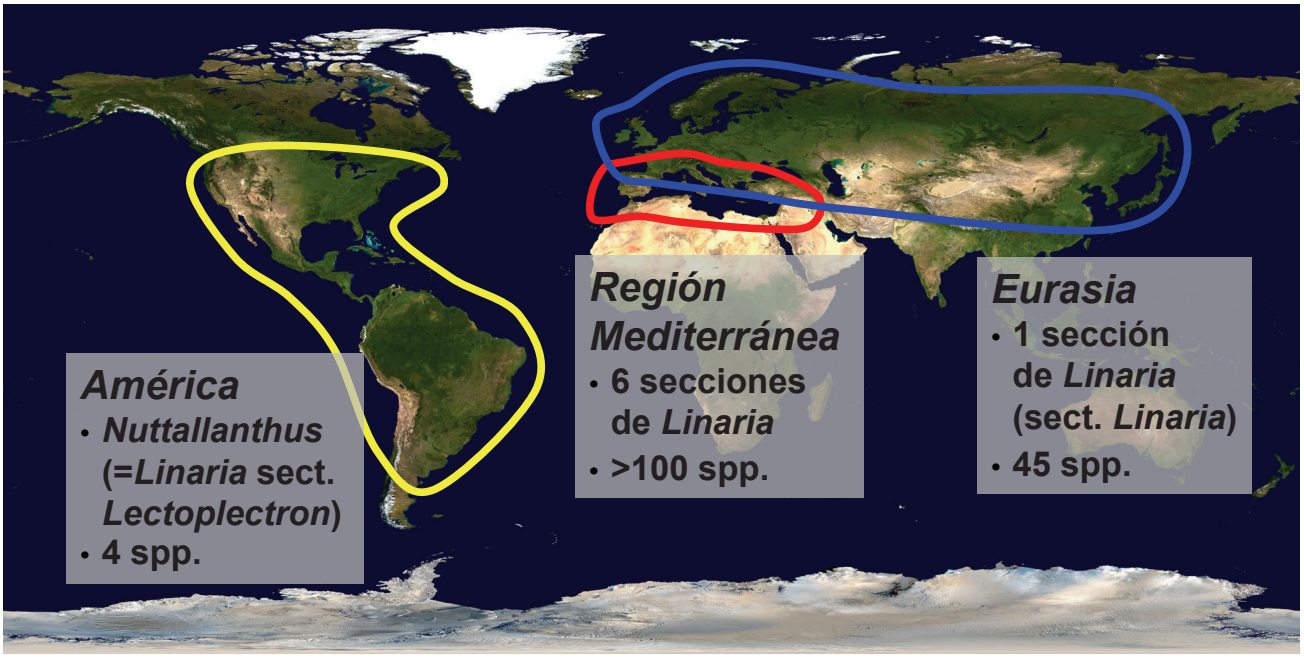


Fig. 5. Distribución geográfica de las secciones y especies de *Linaria*. Se incluyen también las especies americanas de *Nuttallanthus*.

filogenéticas, acompañadas de reconstrucciones biogeográficas y datación, tiene el potencial de resolver esta cuestión (Apéndice 1; Capítulo 2).

La sect. *Versicolores* tiene su centro de diversidad en el Mediterráneo occidental. La mayor diversidad se encuentra en el noroeste de África (Marruecos, Argelia y Túnez, unas 15 especies y subespecies), aunque también hay un buen número de táxones en la península Ibérica (España y Portugal, unas 12 especies y subespecies) (Tabla 3; Viano, 1978b, c; Sutton, 1988). Tres especies se distribuyen a ambos lados del estrecho de Gibraltar: *L. incarnata*, *L. pedunculata* y *L. gharbensis* (Viano, 1969, 1978b; Sáez & Bernal, 2009). *L. multicaulis* se encuentra ampliamente distribuida por el noroeste de África, desde Marruecos hasta Túnez, y alcanza, además, la isla de Sicilia y el extremo sur de la península Itálica (región de Calabria) (Sutton, 1988; De Leonardis *et al.*, 2003). *L. hellenica* es un endemismo de la costa meridional de Grecia (Contandriopoulos & Yannitsaros, 1975) que algunos autores (Tan & Iatrou, 2001) han considerado asimilable a *L. tenuis*. Ésta última es la especie más ampliamente distribuida de la sect. *Versicolores*, con un área que se extiende por el noreste de África (Túnez, Libia, Egipto) y alcanza las estepas del suroeste de Asia (Palestina, Siria, Irak, Irán) (Sutton, 1988). Las áreas de distribución descritas proporcionan un caso interesante para el estudio de los patrones de colonización entre Europa

y África, como ya se ha hecho en otros géneros (Fernández-Mazuecos & Vargas, 2010). Dado que la herencia del plasto es materna en *Linaria* (Corriveau & Coleman, 1988), el genoma plastidial se dispersa únicamente por medio de las semillas. Como la colonización de nuevos territorios ocurre mediante semillas, el ADN plastidial proporciona los marcadores idóneos para la reconstrucción de los patrones de colonización (Petit *et al.*, 1993; Petit *et al.*, 2003). Este planteamiento se aplica a *Linaria* sect. *Versicolores* en el Capítulo 3.

Genética de poblaciones y filogeografía

Se han publicado escasos estudios de genética de poblaciones de especies de *Linaria*. Por medio de aloenzimas, Segarra-Moragues & Mateu-Andrés (2007) estudiaron el efecto del sistema de reproducción en la estructura genética de varias especies de la sect. *Supinae*. De acuerdo con las expectativas, se encontró una menor diversidad genética y mayor diferenciación poblacional en las especies autógamas que en las autoincompatibles. Mediante la aplicación de la misma técnica, Crawford & Elisens (2006) encontraron una baja diversidad y elevada diferenciación en tres especies autógamas de *Nuttallanthus*. Merece la pena mencionar también dos estudios de ISSRs (*Inter Simple Sequence Repeats*) efectuados en poblaciones invasoras de *L. vulgaris* y *L. dalmatica* (Ward *et al.*, 2008; Ward *et al.*, 2009).

Por el momento no se ha publicado ningún estudio filogeográfico del género *Linaria*. El análisis en un contexto geográfico de la diversidad de secuencias de ADN dentro de una especie (o en especies próximas) puede proporcionar una información esencial de cara a reconstruir la historia de las especies (Avise, 2009). En particular, el papel de las penínsulas del sur de Europa como refugios glaciares durante el Cuaternario está siendo objeto de numerosos estudios (Gómez & Lunt, 2006; Nieto-Feliner, 2011). La historia de la distribución de *L. elegans*, actualmente un anillo de montañas en la mitad norte de la península Ibérica, se aborda en el Capítulo 5 mediante métodos filogeográficos, acompañados de técnicas de modelización de la distribución de especies (Elith *et al.*, 2006).

Biología de la conservación

Aunque algunas especies de *Linaria* son comunes y de amplia distribución (como *L. vulgaris*), otras son endemismos restringidos a unas pocas poblaciones que merecen, por tanto, atención desde el punto de vista de la biología de la conservación. Algunas especies reciben protección legal a nivel europeo al estar incluidas en la Directiva de Hábitats, y otras se encuentran protegidas por las diversas legislaciones nacionales o regionales. En España, hay que destacar varios proyectos y líneas de investigación enfocados a la biología de la conservación de especies como *L. lamarckii* (Plaza & Rodríguez, 2009), *L. orbensis* (Herreros *et al.*, 2009) y *L. nigricans* (Benito *et al.*, 2009; Peñas *et al.*, 2011).

Cuatro especies de la sect. *Versicolores* se encuentran catalogadas en la Lista Roja de la flora vascular española (Moreno, 2008), que asigna categorías de amenaza de acuerdo con la clasificación de la Unión Internacional para la Conservación de la Naturaleza[2] (UICN): *L. nigricans* y *L. gharbensis* con la categoría EN y *L. clementei* y *L. pedunculata* con la categoría VU. Además, recientemente se ha asignado la categoría NT a *L. salzmannii* (Sáez & Sainz, 2009). Ninguna especie se ha incluido en el Catálogo Español de Especies Amenazadas (que implica protección legal), pero dos –*L. nigricans* y *L. clementei*– se han incluido en el Catálogo Andaluz con la categoría de "vulnerables". El endemismo griego *L. hellenica* y el portugués *L. algarviana* están protegidos por el anexo II de la Directiva de Hábitats. El último recibió la categoría VU (Walter & Gillett, 1997), aunque recientemente se ha rebajado a LC (Barreto Caldas, 2011). En el norte de África, hasta donde sabemos no se ha realizado una valoración del estado de conservación de las especies de *Linaria* sect. *Versicolores* según las categorías de la UICN. Sin embargo, Fennane & Ibn Tattou (1998) sí incluyen varias especies y subespecies en su catálogo de plantas vasculares raras, amenazadas o endémicas de Marruecos, designando cinco de ellas como "muy raras": *L. dissita*, *L. gattefossei*, *L. pedunculata*, *L. multicaulis* subsp. *gigantea* y *L. viviesiae*; y tres más como "raras": *L. weilleri*, *L. maroccana* y *L. tingitana* (las dos últimas con dudas).

L. nigricans es la especie de *Linaria* sect. *Versicolores* que ha recibido mayor atención conservacionista, al ser representativa de un tipo de hábitat muy peculiar a nivel europeo, los

[2] Las categorías mencionadas en el texto son las siguientes: EN, en peligro; VU, vulnerable; NT, casi amenazada; LC, preocupación menor.

subdesiertos del sureste español. Estudios de modelización han mostrado que la fragmentación de su hábitat debida a la expansión de los cultivos en invernadero en esta región pone en riesgo gravemente su supervivencia a medio plazo (Benito *et al.*, 2009; Peñas *et al.*, 2011).

La otra cara de la moneda son las especies de *Linaria* que se han convertido en invasoras en ciertos países fuera de su área nativa, en particular *L. vulgaris* y *L. dalmatica*. Se están dedicando intensos esfuerzos e investigación para su control y erradicación, particularmente en Estados Unidos (Pauchard *et al.*, 2003; Peterson *et al.*, 2005; Sing & Peterson, 2011).

Registro fósil

El carácter herbáceo de todas las especies de *Linaria* dificulta su fosilización, por lo que el registro de macrofósiles del género es muy escaso. Tampoco es abundante el registro polínico, al tratarse de especies de polinización entomófila (por tanto, con una producción reducida de polen) y, en general, poco abundantes en las comunidades vegetales. La mayoría de los fósiles de *Linaria* que hemos podido localizar en la bibliografía (Tabla 5; Fig. 6) son semillas, y se enmarcan en un intervalo de tiempo reciente. Los dos posibles fósiles más antiguos corresponden al Plioceno superior de Rusia y Groenlandia (Dorofeev, 1963; Bennike & Böcher, 1990), aunque su adscripción taxonómica es incierta. Más frecuentes e inequívocos son los hallazgos de semillas aladas de *Linaria* (identificadas en todos los casos, de manera algo aventurada, como pertenecientes a *L. vulgaris*) en sedimentos del Pleistoceno de Gran Bretaña y Centroeuropa (Allison *et al.*, 1952; Godwin, 1975; Szczepanek & Stachowicz-Rybka, 2004). Más allá de confirmar el carácter nativo de *L. vulgaris* (u otras especies próximas de la sect. *Linaria*) en estas regiones y su posible supervivencia durante periodos glaciares, estos registros son demasiado recientes como para aportar información acerca de la historia evolutiva del género. Por el mismo motivo, tampoco son susceptibles de utilización para la calibración temporal de análisis filogenéticos datados. Para éstos necesitaremos filogenias datadas en un contexto filogenético más amplio (géneros y familias próximos para los cuáles sí se han encontrado fósiles antiguos), que nos permitirán estimar la edad de divergencia del género *Linaria* con otros cercanos. A partir de estas edades podremos, a su vez, estimar los tiempos de divergencia dentro del género *Linaria* (véanse Apéndice 1 y Capítulos 3 al 5).

Tabla 5. Registro fósil del género *Linaria*. Las entradas están ordenadas por antigüedad.

Taxon	Estructura	Edad	Periodo	Localidad	Referencia
cf. *Linaria* sp.	Desconocida	2,5–2,0 Ma	Plioceno superior	Kap Køzrbenhav, Groenlandia	Bennike & Böcher (1990), Funder *et al.* (2001)
Linaria vulgaris	Semilla/fruto	3,6–1,81 Ma	Plioceno superior	Chernoluch, Rusia	Dorofeev (1963)
cf. *Linaria vulgaris*	Semillas	c. 400.000 años	Pleistoceno medio	Clacton, Reino Unido	Godwin (1975)
Linaria vulgaris	Semillas	c. 120.000 años?	Pleistoceno superior	Cambridge, Reino Unido	Lambert *et al.* (1963)
Linaria vulgaris	Fruto	c. 42.000 años	Pleistoceno superior	Earith, Reino Unido	Bell (1970)
Linaria vulgaris	Semillas	> 20.000 años	Pleistoceno superior	Nazeing, Reino Unido	Allison *et al.* (1952)
Linaria vulgaris	Semillas	c. 12.000 años	Pleistoceno superior	Nant Ffrancon, Reino Unido	Burrows (1974)
Linaria vulgaris	Semillas	c. 12.000 años	Pleistoceno superior	Elstead, Reino Unido	Seagrief & Godwin (1960)
Linaria vulgaris	Semillas	c. 12.000 años	Pleistoceno superior	Hartford, Reino Unido	Godwin (1959)
Linaria sp.	Polen	c. 12.000 años	Pleistoceno superior	Esthwaite Basin, Reino Unido	Franks & Pennington (1961)
Linaria vulgaris	Semilla	c. 10.000 años	Pleistoceno superior	Silesia, Polonia	Szczepanek & Stachowicz-Rybka (2004)
cf. *Linaria* sp.	Polen	c. 10.000 años	Pleistoceno superior	Bamburgh, Reino Unido	Bartley (1966)
Linaria vulgaris	Desconocido	7.500 años	Holoceno	Río Svapa, Rusia	Sidorchuk *et al.* (2011)
Linaria vulgaris	Semillas	c. 3.000 años	Holoceno tardío	Río Dudváh, Eslovaquia	Pišút *et al.* (2010)

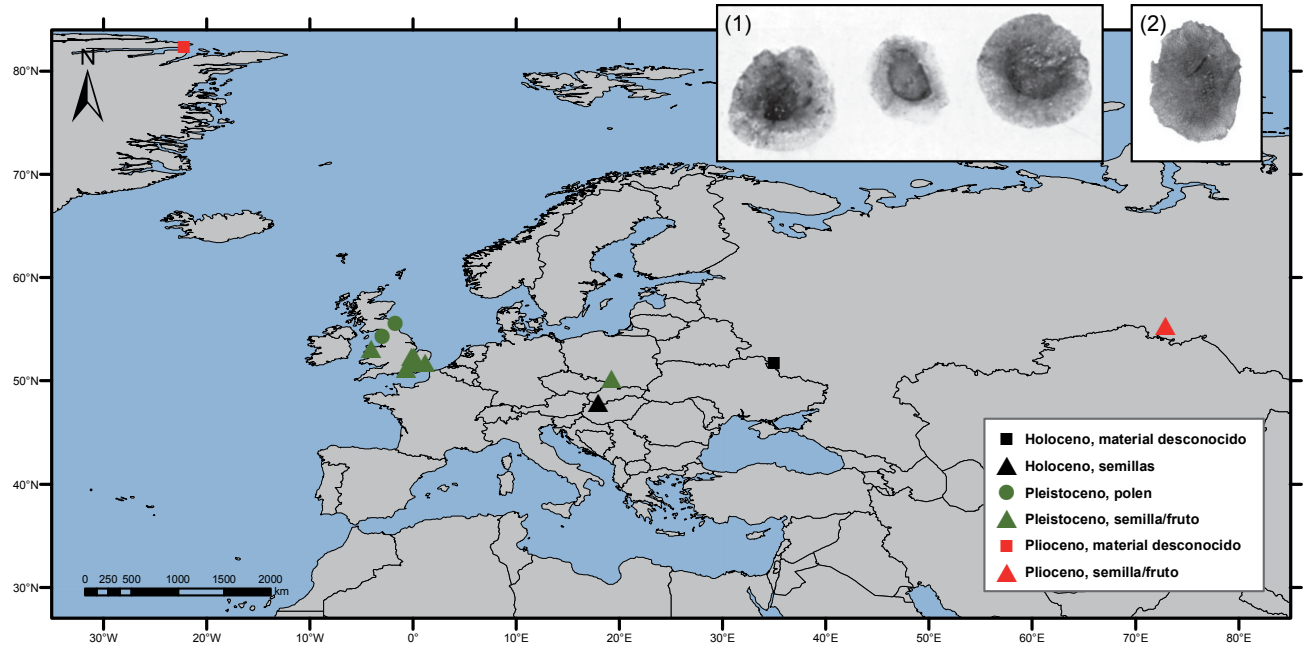


Fig. 6. Distribución geográfica de los fósiles conocidos de *Linaria*. Las fotografías muestran semillas aladas fósiles (*L. vulgaris* según los autores) del Pleistoceno superior de Nazeing (Reino Unido) (1) y de Silesia (Polonia) (2) (Allison *et al.*, 1952; Szczepanek & Stachowicz-Rybka, 2004).

Filogenia y evolución

En las últimas décadas se han formulado diversas hipótesis acerca de la evolución del género *Linaria*, basadas en el análisis comparado de los caracteres morfológicos de las semillas, flores y otras estructuras. Rothmaler (1943) fue el primero en presentar una hipótesis de las relaciones filogenéticas dentro de la tribu Antirrhineae (Fig. 1A). Agrupó el género *Linaria* con otros géneros portadores de espolón: *Cymbalaria*, *Kickxia* y *Chaenorhinum*. Valdés (1970b, d), Viano (1978c, a) y Sutton (1988) propusieron distintas hipótesis sobre la evolución del género. Viano conjeturó una dicotomía basal en la evolución de *Linaria*, de tal forma que las especies de semillas aladas y ápteras constituirían, según esta autora, dos grupos naturales hermanos de divergencia antigua (Fig. 7A). Por el contrario, Valdés y Sutton consideraron que la división del género en dos grupos, basada en la presencia o ausencia de ala en las semillas, tiene un carácter eminentemente práctico, y carece de significado sistemático y evolutivo. Para hacer esta afirmación se basaron en la falta de homología del ala de la semilla de la sect. *Pelisserianae* y de las sects. *Linaria* y *Speciosae*.

Por otro lado, se ha discutido la condición de grupo natural y el significado evolutivo de las distintas secciones de *Linaria* de acuerdo con los estudios morfológicos. Algunas secciones presentan determinados caracteres que son únicos dentro del género (o incluso la tribu), y que se han propuesto como sinapomorfías bajo la hipótesis de que han aparecido una sola vez en la evolución de *Linaria*. Es el caso del estigma bipartito de la sect. *Versicolores*, las semillas comprimidas dorsiventralmente de la sect. *Pelisserianae* y el diminuto sépalo adaxial de la sect. *Macrocentrum*. Otras secciones (*Linaria*, *Speciosae*, *Supinae*) se definen por conjuntos de caracteres, tales como el hábito, el tipo de semillas y la forma de los tallos, que, sin ser sinapomorfías, les hacen parecer también grupos naturales. Finalmente, Valdés y Sutton han afirmado que la sección *Diffusae* es probablemente un grupo polifilético, un "cajón de sastre" en el que se ha incluido un conjunto de especies de semillas ápteras que no parecían encajar en otras secciones, pero que tampoco se encuentran necesariamente relacionadas entre sí.

La sección *Versicolores* constituye uno de los grupos morfológicamente mejor definidos del género *Linaria*, por lo que se ha propuesto que constituye un grupo monofilético, descendiente

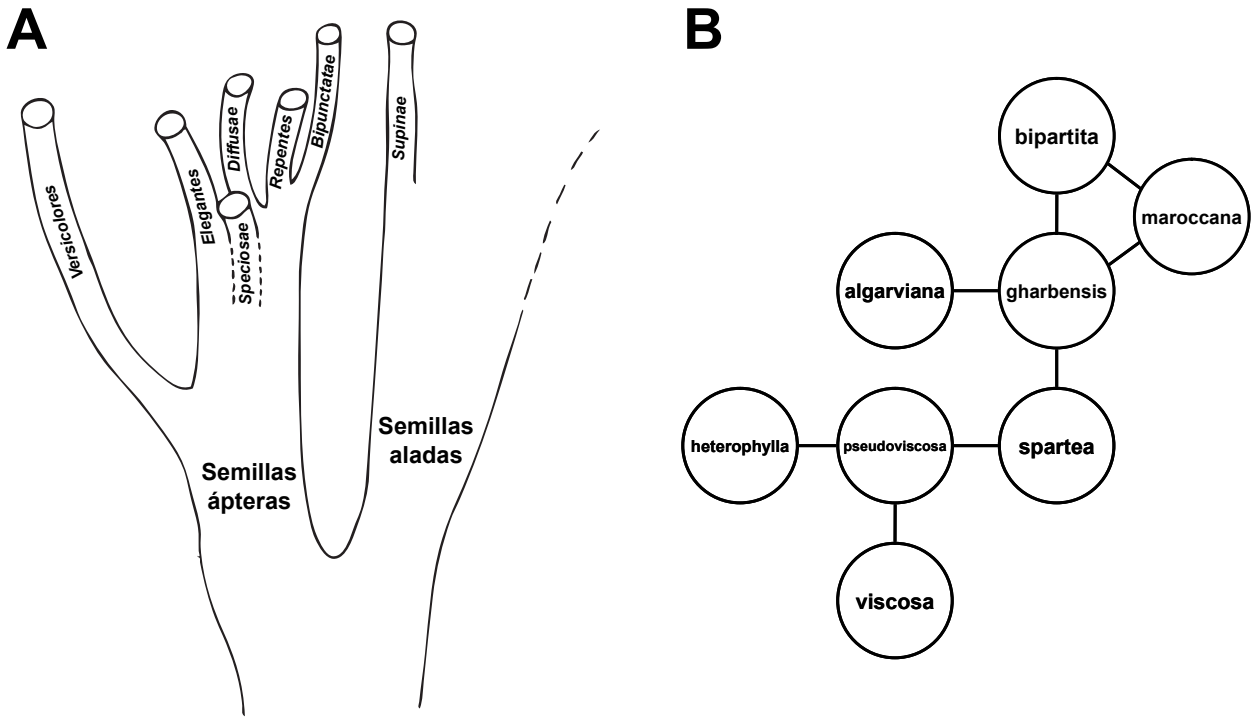


Fig. 7. (A) Hipótesis de relaciones evolutivas entre secciones de *Linaria* según Viano (1978c). (B) Hipótesis de relaciones genéticas entre especies de la subsect. *Versicolores* basada en experimentos de hibridación (Viano, 1978a).

de un único antepasado común (Sutton, 1988). Esta hipótesis se basa en la presencia de una hipotética sinapomorfía, el estilo dividido con dos áreas estigmáticas separadas, que no aparece en ningún otro grupo. En cuanto a la posición evolutiva del linaje de *Versicolores* en relación a otros del género *Linaria*, Viano (1978c) propuso una posición basal de su sect. *Versicolores* (= subsect. *Versicolores*) dentro de un hipotético linaje de semillas ápteras, aunque interpretó el estigma bífido, la forma de la semilla y la predominancia de la anualidad en *Versicolores* como caracteres derivados. La aptitud para la hibridación (escasas barreras reproductivas) dentro de la subsect. *Versicolores* hace suponer que se trata de un grupo de especiación reciente (Viano, 1978a, c), al igual que otras secciones de *Linaria* (Valdés, 1970d). Los experimentos de hibridación de Viano (1978a) permitieron a esta autora proponer un esquema hipotético de "relaciones genéticas" entre especies (Fig. 7B).

El análisis de secuencias de ADN permite poner a prueba las hipótesis comentadas. Por el momento, el único análisis filogenético que ha incluido un muestreo representativo de especies de *Linaria* es la filogenia de Antirrhineae de Vargas *et al.* (2004), basada en secuencias nucleares

ITS (Fig. 1B). Este análisis incluyó ocho especies, con al menos un representante de cada sección reconocida por Sutton (1988). Estas especies formaron un grupo monofilético bien apoyado, pero de relaciones inciertas con otros linajes de antirrhineas. Además, un análisis anterior de la tribu Antirrhineae basado en secuencias plastidiales (Ghebrehiwet *et al.*, 2000), si bien con un muestreo limitado, mostró para el género *Linaria* unas relaciones de parentesco incongruentes con las obtenidas utilizando secuencias nucleares (véase Vargas *et al.*, 2004). Dentro del clado de *Linaria* obtenido por Vargas *et al.* (2004), se obtuvo escasa resolución. El único clado apoyado fue el formado por los representantes de las sects. *Supinae* y *Diffusae*. Dado que no se incluyó en el análisis ningún representante de *Nuttallanthus*, no se pudo evaluar el parentesco entre este género y *Linaria*. En el Capítulo 2 de esta memoria se presenta un análisis filogenético ampliado de la tribu Antirrhineae, que incluye un muestreo mucho más profundo de secuencias ITS de todas las secciones de *Linaria*, así como de *Nuttallanthus*. Esto permitirá analizar en detalle la evolución del tipo de semilla y evaluar la naturalidad de las secciones y subsecciones propuestas por distintos autores. Asimismo, en el Apéndice 2 se presenta la primera filogenia del género basada en secuencias del ADN plastidial. En el Capítulo 4 se analizará en detalle la historia evolutiva de la sect. *Versicolores*. En particular, se estudiará la evolución de ciertos caracteres florales, así como su influencia sobre la tasa de diversificación del clado.

Usos

Muchas especies de *Linaria* se utilizan como plantas ornamentales por su valor estético, como *L. vulgaris*, *L. repens*, *L. dalmatica* y *L. purpurea*. De la sect. *Versicolores*, se utilizan distintas variedades frecuentemente designadas como *L. maroccana* hort. Por sus caracteres morfológicos, estas plantas no se corresponden con la *L. maroccana* Hook.f. endémica del Alto Atlas marroquí. Más bien parecen ser híbridos complejos en los que podrían estar implicadas distintas especies marroquíes, como *L. gharbensis*, *L. bipartita*, *L. maroccana* o *L. incarnata* (Maire, 1941; Sutton, 1988). Por otro lado, debido a la presencia de ciertos compuestos químicos, se han atribuido a algunas especies propiedades medicinales, como antioxidantes y antitumorales (Tundis *et al.*, 2005; Vrchovská *et al.*, 2008)

Justificación y objetivos

Como se ha visto, en los dos últimos siglos se ha acumulado una gran cantidad de información sobre la biología del género *Linaria*. Aún no disponemos, sin embargo, de una hipótesis robusta de relaciones filogenéticas que sirva de marco evolutivo y espacio-temporal para estos estudios. Un primer objetivo de esta memoria doctoral ha sido proporcionar dicho marco por medio de técnicas y métodos filogenéticos basados en secuencias de ADN. Se ha otorgado especial énfasis al estudio de un grupo de especies de *Linaria*, la sect. *Versicolores*, para la cual se planteó como hipótesis inicial de trabajo su monofilia. En primer lugar se sometió a test la monofilia de este grupo de especies, definido según la monografía más reciente de la sección (Sutton, 1988). Una vez se confirmó que se trata de un linaje bien definido, se sometió a prueba la siguiente **hipótesis general**: la evolución de *Linaria* sect. *Versicolores* ha estado determinada tanto por factores históricos abióticos (movimientos de las placas tectónicas, cambios climáticos), como por factores bióticos (en particular los insectos polinizadores). Así, se ha planteado una serie de hipótesis explícitas de tipo biogeográfico, filogeográfico y evolutivo, las cuales se han puesto a prueba mediante el análisis de secuencias de ADN, complementadas con conjuntos de datos de caracteres morfológicos, ecológicos y de distribución. En concreto, el análisis de secuencias de los genomas nuclear y plastidial se ha complementado con la información proporcionada por la revisión taxonómica, los análisis morfométricos, los censos de polinizadores y la modelización de la distribución de especies. Se ha considerado que el mejor planteamiento para reconstruir la historia evolutiva de *Linaria* sect. *Versicolores* es estructurar el presente estudio a varias escalas macro- y microevolutivas, desde la más amplia escala de tribu (Antirrhineae), pasando por estudios a nivel de género (*Linaria*) y sección (*Versicolores*), hasta el nivel de poblaciones de una misma especie (*L. elegans*).

Los **objetivos particulares** propuestos son los siguientes:

1. Evaluar las relaciones filogenéticas del género *Linaria* dentro de la tribu Antirrhineae, su monofilia y su relación de parentesco con el género *Nuttallanthus* (Capítulo 2).

2. Evaluar la monofilia de las secciones y subsecciones reconocidas por distintos autores dentro del género *Linaria*, en particular de la sección *Versicolores* y, dentro de ella, de las subsecciones *Versicolores* y *Elegantes* (Capítulo 2).

3. Analizar la evolución de los caracteres seminales a lo largo de la filogenia de *Linaria*; en concreto, reconstruir los cambios evolutivos entre semillas aladas y no aladas (Capítulo 2).

4. Inferir las relaciones filogenéticas dentro de *Linaria* sect. *Versicolores* a partir del análisis de secuencias nucleares y plastidiales (Capítulo 3, Capítulo 4).

5. Datar la diversificación de *Linaria* sect. *Versicolores* y reconstruir así los tiempos absolutos de divergencia en la historia evolutiva de los diferentes linajes (Capítulo 3, Capítulo 4).

6. Reconstruir y datar los patrones de colonización de *Linaria* sect. *Versicolores* a lo largo y ancho de la cuenca Mediterránea; en particular, evaluar el papel del mar Mediterráneo como barrera biogeográfica asociada a eventos de dispersión y/o vicarianza entre el sur de Europa y el norte de África (Capítulo 3).

7. Caracterizar la diversidad morfológica floral en *Linaria* sect. *Versicolores*, y reconstruir su evolución y su efecto sobre las tasas de diversificación (especiación y extinción) del grupo (Capítulo 4).

8. Caracterizar la fauna polinizadora en una muestra representativa de especies de *Linaria* sect. *Versicolores* y evaluar su influencia sobre los cambios morfológicos florales más notables (Capítulo 4).

9. Reconstruir la historia evolutiva y de colonización de una especie montana de *Linaria* sect. *Versicolores* (*L. elegans*) mediante reconstrucciones filogeográficas, dataciones y modelización de la distribución. En concreto, se pondrán a prueba distintas hipótesis acerca del efecto de la sucesión de periodos glaciares e interglaciares del Cuaternario sobre la distribución de la especie (Capítulo 5).

Estructura de la memoria doctoral

La presente memoria se ha estructurado en cuatro capítulos principales y cuatro apéndices. En los capítulos se presenta la investigación original llevada a cabo por el doctorando y que se dirige al contraste de la hipótesis general y al cumplimiento de los objetivos arriba enumerados. En los apéndices se presenta investigación original a la que el doctorando ha contribuido significativamente y que, sin estar enfocada directamente al cumplimiento de los objetivos, sí es relevante para ese fin y para la correcta comprensión de los capítulos. Los capítulos y anexos se enumeran a continuación:

- En el **Capítulo 2** se presenta un análisis filogenético del género *Linaria* basado en secuencias nucleares ITS y en el marco de una filogenia de la tribu Antirrhineae. Se aplican métodos de contraste de hipótesis y de reconstrucción de caracteres para evaluar el parentesco entre *Linaria* y *Nuttallanthus*, la naturalidad de la taxonomía infragenérica y la evolución de los caracteres morfológicos de la semilla.

- En el **Capítulo 3** se presenta un análisis filogenético de *Linaria* sect. *Versicolores* basado en secuencias del ADN plastidial. Se aplican métodos de datación, reconstrucción biogeográfica y filogeografía para la reconstrucción de los patrones de colonización del grupo en un marco temporal.

- En el **Capítulo 4** se presenta una filogenia datada de *Linaria* sect. *Versicolores* basada en secuencias nucleares y plastidiales y obtenida mediante métodos recientemente desarrollados basados en la teoría de la coalescencia. Se utilizan técnicas de morfometría tradicional y morfometría geométrica para caracterizar la variación en la forma de las flores, y se analiza la evolución de ésta a lo largo de la filogenia mediante los métodos y técnicas del análisis comparativo: reconstrucción de caracteres, correlaciones y efecto de los caracteres sobre las tasas de especiación y extinción. También se presentan los primeros datos sobre visitantes florales de la sect. *Versicolores*, y se evalúa el papel de éstos sobre la evolución de los rasgos florales.

- En el **Capítulo 5** se ponen a prueba las tres hipótesis más plausibles acerca de la historia evolutiva durante el Cuaternario de *L. elegans*, una especie distribuida principalmente por

un anillo de montañas en la mitad norte de la península Ibérica. Para ello se utilizan dos tipos de métodos: por un lado, se efectúa un estudio filogeográfico basado en secuencias nucleares y plastidiales y en análisis recientemente desarrollados dentro de la teoría de la coalescencia; por otro, se modeliza la distribución de la especie mediante el método de la máxima entropía.

- En el **Capítulo 6** se discuten en conjunto los resultados de la memoria, y se extraen de ellos conclusiones generales, en particular las relativas a la hipótesis general de partida.

- En el **Apéndice 1** se presenta una filogenia datada de la tribu Antirrhineae basada en secuencias plastidiales y calibrada con una serie de fósiles del orden Lamiales y una calibración secundaria. Las edades de divergencia obtenidas en este análisis han sido utilizadas para la datación de los análisis filogenéticos y filogeográficos de *Linaria* sect. *Versicolores* presentados en los Capítulos 3, 4 y 5.

- En el **Apéndice 2** se presenta un análisis filogenético del género *Linaria* basado en secuencias del ADN plastidial. Este análisis complementa el análisis basado en secuencias nucleares del Capítulo 2. También sirve de apoyo al Capítulo 3, al poner a prueba la monofilia de la sect. *Versicolores* utilizando marcadores de herencia materna (plastidiales).

- En el **Apéndice 3** se lleva a cabo una revisión taxonómica del complejo ibero-norteafricano *L. incarnata*. Esta revisión se consideró necesaria con el fin de obtener una delimitación de especies robusta de cara a los análisis filogenéticos y de diversificación del Capítulo 4. Se describe una especie nueva para la ciencia, *L. mamorensis*, y se recupera otra, *L. onubensis*, no reconocida por autores recientes.

- En el **Apéndice 4** se presenta un análisis filogeográfico preliminar de un linaje ibérico de *Linaria* sect. *Versicolores*. Este estudio sirve de complemento al Capítulo 3, al ayudar a resolver las relaciones dentro de un clado escasamente resuelto con métodos filogenéticos.

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Capítulo 2

Análisis filogenético del género *Linaria* basado en secuencias nucleares ITS: consecuencias para la sistemática y la evolución de los caracteres seminales

A phylogeny of toadflaxes (*Linaria* Mill.) based on nuclear ITS sequences: consequences for systematics and evolution of seed shape

Este capítulo se ha desarrollado en colaboración con José Luis Blanco Pastor (Real Jardín Botánico, CSIC).

Una versión de este capítulo ha sido aceptada para su publicación en *International Journal of Plant Sciences*:

Fernández-Mazuecos M, Blanco-Pastor JL, Vargas P. A phylogeny of toadflaxes (*Linaria* Mill.) based on nuclear ITS sequences: systematic and evolutionary consequences. *International Journal of Plant Sciences*. Aceptado.

Abstract

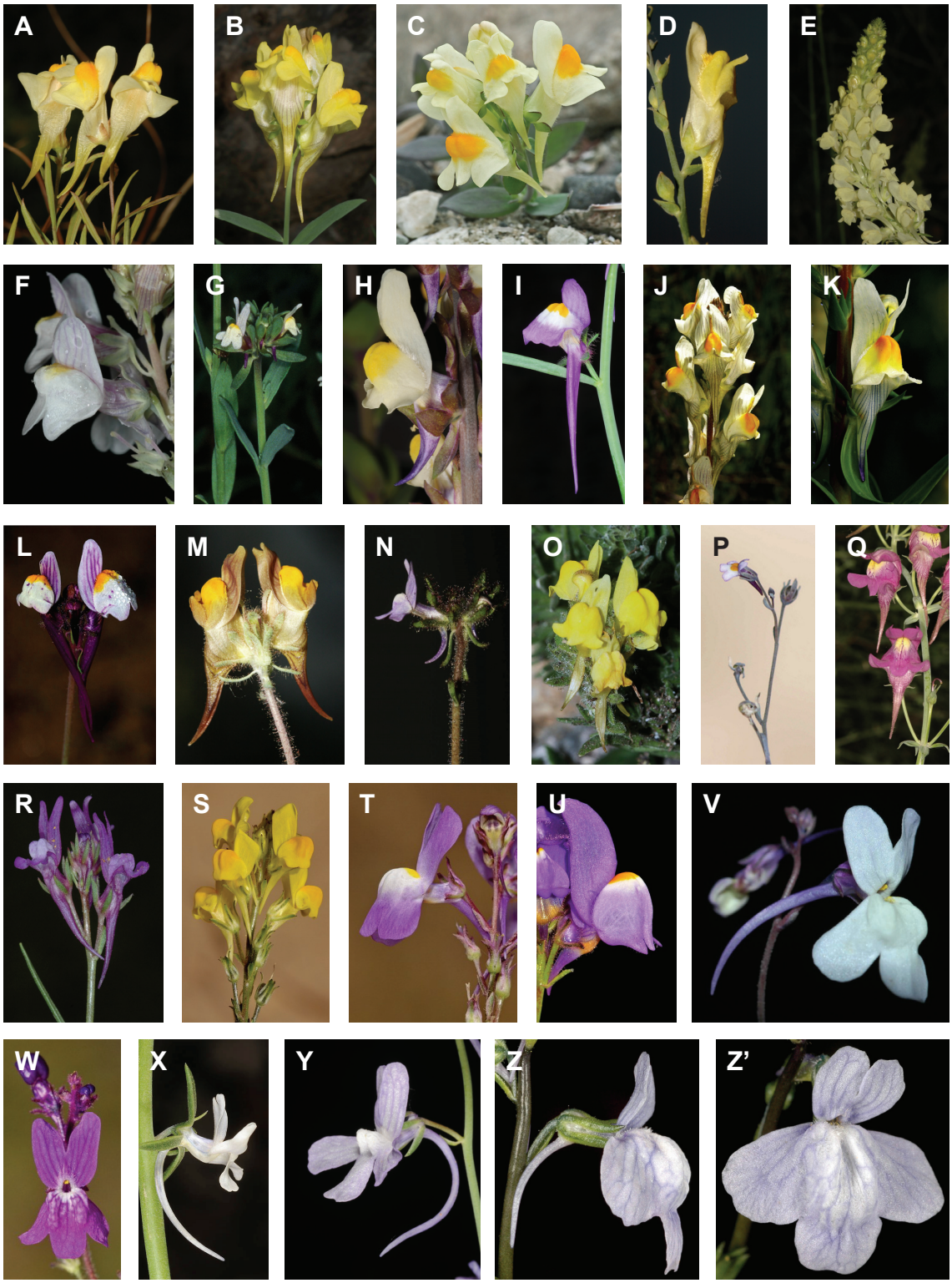
Toadflaxes (*Linaria* Mill., c. 150 spp. from the Palearctic region) constitute the largest genus of the snapdragon lineage (tribe Antirrhineae). Here we provide the first extensive phylogenetic testing of systematic and evolutionary hypotheses about toadflaxes. Internal transcribed spacer (ITS) sequences were obtained for 94 species representing all sections of *Linaria* recognized by recent taxonomic treatments, as well as three species of the morphologically related American genus *Nuttallanthus*. In addition, 71 sequences representing the remaining 26 genera of Antirrhineae were gathered to test the monophyly of *Linaria*. Phylogenetic analyses were conducted using Bayesian inference, maximum likelihood and maximum parsimony. The evolution of seed morphology was investigated through ancestral state reconstruction methods. *Linaria* and *Nuttallanthus* constituted a strongly-supported monophyletic group within the Antirrhineae. *Linaria* was revealed as a paraphyletic group, with *Nuttallanthus* nested within it. Six major clades were recognized within *Linaria* (*s.l.*). A seed dispersal structure (seed wing), which has been extensively used in systematic treatments, appears to have arisen multiple times in the course of the evolution of *Linaria*. The circumscription of *Nuttallanthus* within *Linaria* is suggested in order to achieve the monophyly of the latter genus. Some sections of *Linaria* (*Macrocentrum*, *Pelisserianae* and *Versicolores*) which are well-defined by distinct morphological traits are also supported as natural groups, while monophyly of the remaining sections (*Supinae*, *Linaria*, *Speciosae* and *Diffusae*) is unsupported. Habit, inflorescence and flower morphology, coupled with seed morphology, are revealed as the key characters in the evolution of toadflaxes.

Introduction

One hundred and fifty species of toadflaxes (*Linaria* Mill.; see examples in Fig. 1) were proposed in the last taxonomic treatment of the tribe Antirrhineae (Sutton, 1988). *Linaria* constitutes the largest of the 28 genera of this tribe, and displays the key flower characteristics of *Antirrhinum*, a model group for plant developmental and evolutionary research (Schwarz-Sommer *et al.*, 2003). The personate, spurred, usually occluded corolla of *Linaria* has also attracted attention as a model to understand the development and evolution of floral symmetry (Cubas *et al.*, 1999), flower colour (Galego & Almeida, 2007) and nectar spurs (Box *et al.*, 2011). Active research is also being undertaken on reproductive strategies and pollination (Sánchez-Lafuente *et al.*, 2011), phytochemistry (Beninger *et al.*, 2009), biogeography (Fernández-Mazuecos & Vargas, 2011; Chapter 3) and invasion biology (Sing & Peterson, 2011). Despite this research attention, a well-supported evolutionary framework for *Linaria* research is still lacking.

Linaria was recognized as a taxonomic entity as early as the time of pre-Linnaean botanists (Morison, 1680; Tournefort, 1700). Linnaeus (1753) included previously recognized *Linaria* species within his genus *Antirrhinum*. However, *Linaria* was shortly after accepted as a distinct genus by Miller (1754), who provided the first valid description of the genus. Early authors generally considered the genus *Linaria* in a wide sense, including all those species related to *Antirrhinum* that display a spurred corolla (Lamarck & De Candolle, 1805; Chavannes, 1833; Bentham, 1846). Chavannes (1833) delimited four sections within *Linaria* (*Chaenorhinum*, *Cymbalaria*, *Elatinoides* and *Linariastrum*), which were later separated as distinct genera by Wettstein (1895), giving rise to the current circumscription of *Linaria* (formerly section *Linariastrum*). Only species with entire, sessile leaves and terminal, racemose inflorescences remained under this genus. This view has been adopted by all recent taxonomic treatments (Rothmaler, 1943; Valdés, 1970; Sutton, 1988; Sáez & Bernal, 2009), and has been supported by molecular phylogenetics (Ghebrehiwet *et al.*, 2000; Vargas *et al.*, 2004).

Subdivision of *Linaria* (in its currently accepted sense) has historically followed two main trends (reviewed by Valdés, 1970; Sutton, 1988). A first subdivision into two groups has been based on the presence or absence of an encircling wing in seeds (Fig. 2). This approach seems to date back to Morison (1680), and was widely followed by later authors, but it has rarely been reflected in formal infrageneric taxa (Lange, 1870; Wettstein, 1895; Valdés, 1970). Dumortier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(1827) did distinguish the sections *Leontorrhinum* (winged seeds) and *Lycorrhinum* (wingless seeds), while Boissier (1879) called these groups *Discoideae* and *Oblongae*, respectively. Viano (1978b) hypothesized that species with winged and wingless seeds constitute two sister evolutionary lineages. However, despite being of practical value, this two-partite classification has been considered artificial by Valdés (1970) and Sutton (1988) based on the non-homologous anatomy of seed wings across the genus.

A second, multi-partite approach to *Linaria* subdivision is based on a wider range of vegetative and reproductive traits. This strategy dates back to Chavannes (1833), who recognized five sections. These were named and modified by Bentham (1846) and Wettstein (1895), and constitute the base of subsequent classifications by Valdés (1970), Viano (Viano, 1978a, b) and Sutton (1988) (Table 1). Sutton's classification (Table 2) is widely accepted today, and includes 150 species classified in seven sections: *Linaria* (45 spp.), *Supinae* (44 spp.), *Pelisserianae* (2 spp.), *Versicolores* (21 spp.), *Speciosae* (19 spp.), *Diffusae* (17 spp.) and *Macrocentrum* (2 spp.). The first three sections include species with discoid, usually winged seeds, while the latter four contain species with non-discoid, wingless seeds.

DNA sequences of eight species representing the seven sections of *Linaria* formed a monophyletic group in a previous phylogeny of the tribe Antirrhineae based on the internal transcribed spacer (ITS) region (Vargas *et al.*, 2004). Naturalness of sections is, however, considered doubtful at least in some cases (Valdés, 1970; Sutton, 1988), and it has not been assessed in a molecular phylogenetic framework to date.

Fig. 1. Representatives of *Linaria* and *Nuttallanthus*. *Linaria* sect. *Linaria*: (A) *L. vulgaris*; (B) *L. meyeri*; (C) *L. japonica*. *Linaria* sect. *Speciosae*: (D) *L. dalmatica*; (E) *L. peloponnesiaca*; (F) *L. repens*. *Linaria* sect. *Diffusae*: (G) *L. albifrons*; (H) *L. triphylla*; (I) *L. haelava*; (J) *L. hirta*. *Linaria* sect. *Supinae* subsect. *Supinae*: (K) *L. latifolia*; (L) *L. amethystea*; (M) *L. depauperata*; (N) *L. arvensis*. *Linaria* sect. *Supinae* subsect. *Saxatile*: (O) *L. saxatilis*; (P) *L. tursica*. *Linaria* sect. *Pelisserianae*: (Q) *L. triornithophora*; (R) *L. pelisseriana*. *Linaria* sect. *Versicolores* subsect. *Versicolores*: (S) *L. viscosa*; (T) *L. bipartita*; (U) *L. clementei*. *Linaria* sect. *Versicolores* subsect. *Elegantes*: (V) *L. nigricans*; (W) *L. elegans*. *Linaria* sect. *Macrocentrum*: (X) *L. chalepensis*; (Y) *L. armeniaca*. *Nuttallanthus*: (Z, Z') *N. texanus*. Photos by M. Luceño (A, D, E, F, Q, R), O. Fragman-Sapir (B, G, H, I, X, Y), N. V. Kurzenko (C), J. Ramírez (J, K, L, U), J. L. Blanco-Pastor (M, N, O, P), J. Quiles (S, T, W), P. Vargas (V) and G. D. Carr (Z, Z').

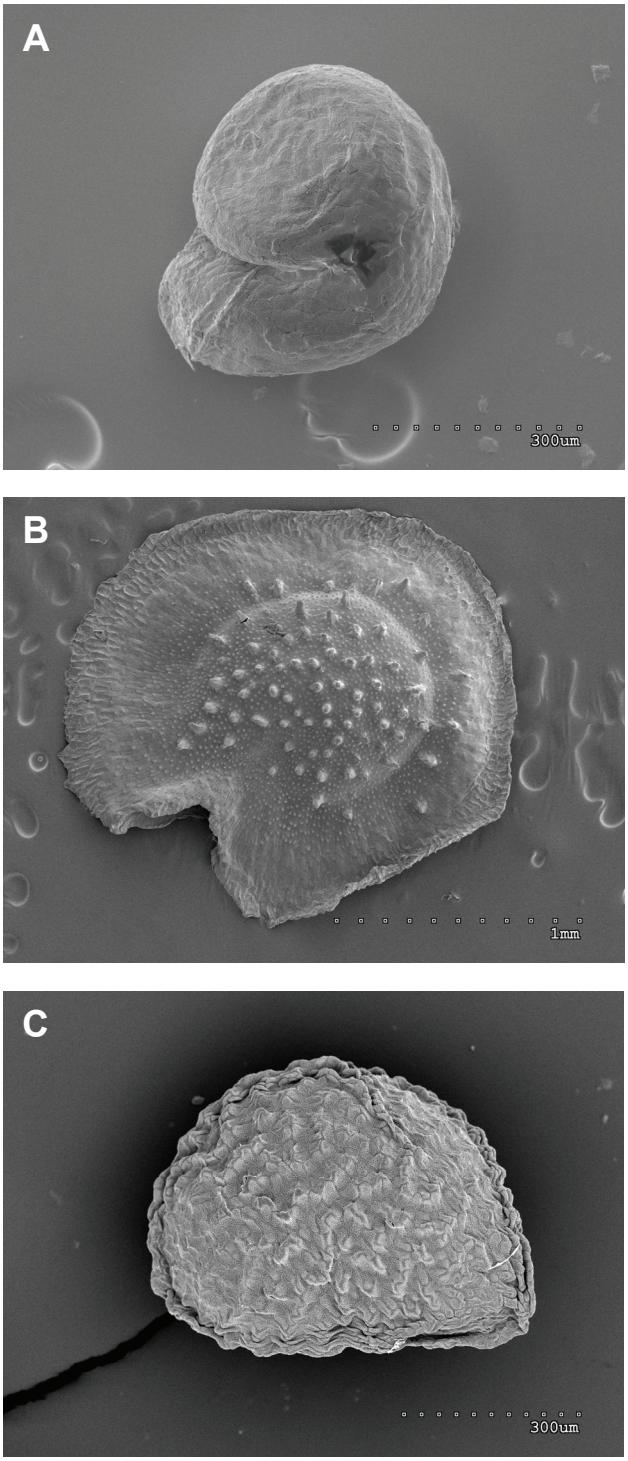


Fig. 2. Examples of *Linaria* seeds belonging to the three major morphological types: (A) no wing (*L. tursica*); (B) wing (*L. simplex*); and (C) marginal ridge (*L. huteri*). Scanning electron microscopy photos by Y. Ruiz.

Table 1. Historical overview of *Linaria* and *Nuttallanthus* classification.

Sutton (1988), Sáez & Bernal (2009) and present study	**Bentham (1846)**	**Wettstein (1895)**	**Valdés (1970) and Viano (1978a, b)**
Linaria Mill.	*Linaria* sect. *Linariastrum* Chav.	*Linaria* Juss.	*Linaria* Mill.
Sect. *Linaria*	§ *Grandes* Benth. p.p.max. § *Supinae* Benth. p.p.min. § *Diffusae* Benth. p.p.min.	Sect. *Grandes* (Benth.) Wettst. p.p.max. Sect. *Supinae* (Benth.) Wettst. p.p.min. Sect. *Diffusae* (Benth.) Wettst. p.p.min.	Sect. *Linaria* p.p.max.
Sect. *Speciosae* (Benth.) Wettst.	§ *Speciosae* Benth. p.p.max. § *Versicolores* Benth. p.p. § *Diffusae* Benth. p.p.min.	Sect. *Speciosae* (Benth.) Wettst. p.p.max. Sect. *Versicolores* (Benth.) Wettst. p.p. Sect. *Diffusae* (Benth.) Wettst. p.p.min.	Sect. *Speciosae* (Benth.) Wettst. p.p. Sect. *Repentes* Valdés ex Viano
Sect. *Diffusae* (Benth.) Wettst.	§ *Diffusae* Benth. p.p. § *Minutiflorae* Benth. § *Speciosae* Benth. p.p.	Sect. *Diffusae* (Benth.) Wettst. p.p. Sect. *Speciosae* (Benth.) Wettst. p.p.	Sect. *Diffusae* (Benth.) Wettst. Sect. *Minutiflorae* Benth. ex Kuprian. Sect. *Speciosae* (Benth.) Wettst. p.p.
Sect. *Supinae* (Benth.) Wettst.			
Subsect. *Supinae*	§ *Arvenses* Benth. p.p.max. § *Supinae* Benth. p.p. § *Diffusae* Benth. p.p. § *Grandes* Benth. p.p.min.	Sect. *Arvenses* (Benth.) Wettst. p.p.max. Sect. *Supinae* (Benth.) Wettst. p.p.	Sect. *Arvenses* (Benth.) Wettst. Sect. *Supinae* (Benth.) Wettst. subsect. *Supinae* Sect. *Supinae* subsect. *Amethystea* Valdés Sect. *Linaria* p.p.min.
Subsect. *Saxatile* Valdés	§ *Supinae* Benth. p.p. § *Versicolores* Benth. p.p.min.	Sect. *Supinae* (Benth.) Wettst. p.p.	Sect. *Supinae* subsect. *Saxatile* Valdés Sect. *Bipunctatae* Viano p.p.max.
Subsect. *Trimerocalyx* (Murb.) D.A.Sutton	Not included	Not included	Not included
Sect. *Pelisserianae* Valdés	§ *Grandes* Benth. p.p.min. § *Arvenses* Benth. p.p.min.	Sect. *Grandes* (Benth.) Wettst. p.p.min. Sect. *Arvenses* (Benth.) Wettst. p.p.min.	Sect. *Pelisseriana* Valdés
Sect. *Versicolores* (Benth.) Wettst.			
Subsect. *Versicolores*	§ *Versicolores* Benth. p.p. § *Diffusae* Benth. p.p.min. § *Supinae* Benth. p.p.min.	Sect. *Versicolores* (Benth.) Wettst. p.p. Sect. *Diffusae* (Benth.) Wettst. p.p.	Sect. *Versicolores* (Benth.) Wettst. Sect. *Bipunctatae* Viano p.p.min.
Subsect. *Elegantes* (Viano) D.A.Sutton	§ *Versicolores* Benth. p.p.min.	Sect. *Versicolores* (Benth.) Wettst. p.p.min.	Sect. *Elegantes* Viano
Sect. *Macrocentrum* D.A.Sutton	§ *Versicolores* Benth. p.p.min.	Sect. *Versicolores* (Benth.) Wettst. p.p.min.	Sect. *Versicolores* (Benth.) Wettst. p.p.min.
Nuttallanthus D.A.Sutton	§ *Versicolores* Benth. p.p.min.	Sect. *Versicolores* (Benth.) Wettst. p.p.min.	Sect. *Lectoplectron* Pennell

Abbreviations: p.p. = pro parte; p.p.max. = pro parte maxima; p.p.min. = pro parte minima.

Table 2. Major features of infrageneric taxa of *Linaria* (according to Sutton, 1988) and *Nuttallanthus*.

	No. species	No. sampled species	Seed shape	Seed wing	Habit	Adaxial lobe of calyx	Stigma	Fruiting calyx	Palate development	Distribution
Linaria										
Sect. *Linaria*	45	9	Discoid, laterally compressed	Wing	Perennial	Normal	Entire	5-partite	Prominent	Eurasia
Sect. *Speciosae*	19	14	Non-discoid	No wing	Perennial	Normal	Entire	5-partite	Prominent	Mediterranean region
Sect. *Diffusae*	17	11	Non-discoid	No wing	Annual or perennial	Normal	Entire	5-partite	Prominent	Mediterranean region
Sect. *Supinae*	44	38								
Subsect. *Supinae*	32	30	Discoid, laterally compressed	Wing or marginal ridge	Annual or perennial	Normal	Entire	5-partite	Prominent	Mediterranean region
Subsect. *Saxatile*	11	8	Non-discoid	Wing, marginal ride or no wing	Annual or perennial	Normal	Entire	5-partite	Prominent	Mediterranean region
Subsect. *Trimerocalyx*	1	0	Discoid, laterally compressed	Wing	Annual	Normal	Entire	3-partite	Prominent	Northern Africa
Sect. *Pelisserianae*	2	2	Discoid, dorsi-ventrally compressed	Wing	Annual or perennial	Normal	Entire	5-partite	Prominent	Mediterranean region
Sect. *Versicolores*	21	19								
Subsect. *Versicolores*	19	17	Non-discoid	No wing	Annual or perennial	Normal	Divided	5-partite	Prominent or weak	Mediterranean region
Subsect. *Elegantes*	2	2	Non-discoid	No wing	Annual	Normal	Emarginate	5-partite	Weak	Iberian Peninsula
Sect. *Macrocentrum*	2	2	Non-discoid	No wing	Annual	Reduced	Entire	5-partite	Weak	Mediterranean region
Nuttallanthus	4	3	Non-discoid	No wing	Annual or biennial	Normal	Entire	5-partite	Weak	North and South America

Linaria is basically a Palearctic genus. It has its diversity centre in the Mediterranean region, where all seven sections of Sutton's classification are present. Only sect. *Linaria* has a wider range that extends over most of Eurasia and reaches the Japanese archipelago. A few toadflax species with wingless seeds native to the New World have historically been circumscribed in different genera. Due to the lack of a well-developed palate they were included in *Anarrhinum* by Desfontaines (1798). More commonly, they have been included in *Linaria*, as part of sect. *Versicolores* (Bentham, 1846; Wettstein, 1895), or as the distinct sect. *Lectoplectron* (Pennell, 1935; Valdés, 1970). Finally, Sutton (1988) transferred these three North American and one South American species to his new genus *Nuttallanthus*, based on flower and seed traits. Molecular phylogenies of Antirrhineae (Ghebrehiwet *et al.*, 2000; Vargas *et al.*, 2004) have not included *Nuttallanthus* accessions to date, and therefore the status of this genus as a distinct evolutionary lineage and its relationships within the tribe Antirrhineae remain unclear.

Here we present the first phylogenetic hypothesis of *Linaria* and *Nuttallanthus* based on nuclear ribosomal DNA sequences. A worldwide sampling of ITS sequences of all Antirrhineae genera, including a wide sample of *Linaria* species and three species of *Nuttallanthus*, has been performed in order to test evolutionary hypotheses proposed by Sutton (1988): (1) that *Nuttallanthus* species constitute an evolutionary lineage distinct from *Linaria*, and are therefore deserving of a separate generic status; and (2) that the seed wing has evolved several times in the course of *Linaria* evolution. In addition, naturalness of the seven sections of Sutton's classification is evaluated for the first time.

Materials and methods

Sampling strategy and DNA sequencing

We sampled plant materials from 94 of c.150 species of *Linaria* and two of four species of *Nuttallanthus* (Table 3). All sections of *Linaria* recognized in recent taxonomic accounts (Table 1; Valdés, 1970; Viano, 1978a, b; Sutton, 1988) were represented. Plant materials were collected in the field and dried in silica gel or obtained from herbarium sheets (MA, RNG, E, ATH). Total genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN Inc., California). We

Table 3. Studied species of *Linaria* and *Nuttallanthus*, with seed wing states, geographic distributions, voucher specimens and GenBank accession numbers.

Taxon	Seed wing state	Distribution	Sampled locality	Voucher	GenBank accession no.
Linaria					
Sect. *Linaria*					
L. angustissima (Loisel.) Borbás	Wing	S and C Europe	Romania, Cluj-Napoca	J. Güemes and G. Bacchetta (MA 618431)	JX481133
L. baligaliensis Patzak	Wing	Afghanistan	Afghanistan, Salong Pass	J.F. Veldkamp (MA 784820)	JX481142
L. japonica Miq.	Wing	E Asia	Japan, Tottori	S. and T. Taniguchi s.n. (MA)	JX481141
L. kurdica Boiss. & Hohen.	Wing	SW Asia	Armenia, Vayots Dzor	A. Herrero *et al.* (MA 744412)	JX481139
L. loeselii Schweigger	Wing	NW Europe	Lithuania, Apskritis ol Klaipeda	E. Glazkova and A. Quintanar (MA 791644)	JX481153
L. meyeri Kuprian.	Wing	Russia	Georgia, Mtskhete Mtianeti	L. Muñoz *et al.* (MA 764400)	JX481150
L. odora (Bieb.) Fisch.	Wing	Russia	Russia, Voilgograd	A.K. Skvortsov (MA 618431)	JX481152
L. thibetica Franch.	Wing	China	China, Sichuan	D.E. Boufford *et al.* (E 00292244)	JX481140
L. vulgaris Mill.	Wing	Eurasia	France, Chamonix	B. Estébanez s.n.	JX481138
Sect. *Speciosae*					
L. antilibanotica Rech.f.	No wing	SW Asia	Lebanon, Baalbek	K. Sleem (RNG)	JX481134
L. capraria Moris & De Not.	No wing	Italy	Italy, Marciana	R. M. Baldini and L. Vivona (MA 693545)	JX481146
L. corifolia Desf.	No wing	SW Asia	Turkey, Dogançal	J. J. Aldasoro (MA 689911)	JX481135
L. creticola Kuprian.	No wing	Russia	Russia, Belgorod	V. Gladkova and T. Leonova (E 00419502)	JX481143
L. dalmatica (L.) Mill.	No wing	SC Europe, SW Asia	Bulgaria, Rhodopes Mountains	C. Navarro *et al.* (MA 726987)	JX481136
L. genistifolia (L.) Mill.	No wing	CE Europe, W Asia	Turkey	J. J. Aldasoro (A9751)	JX481137
L. grandiflora Desf.	No wing	SE Europe, SW Asia	Turkey, Erzurum	A. Herrero *et al.* (MA 687558)	JX481151
L. iconia Boiss. & Heldr.	No wing	Turkey	Turkey, Konya	Gordon C. Hillman (RNG)	JX481154
L. nivea Boiss. & Reut.	No wing	Spain	Spain, Toledo	C. Aedo (MA 611701)	JX481155
L. peloponnesiaca Boiss. & Heldr.	No wing	Balkans	Greece, Mount Olympus	P. Vargas (MA 778352)	JX481148
L. purpurea (L.) Mill.	No wing	Italy	United Kingdom, Norwich (cultivated)	M. Fernández-Mazuecos (74MF09)	JX481147
L. repens (L.) Mill.	No wing	W Europe	Spain, Cuenca, El Tobar	M. Fernández-Mazuecos (54MF09)	JX481144
L. rubioides Vis. & Pančić	No wing	Balkans	Serbia, Mokra Gora	S.L. Jury (RNG)	JX481149
L. ventricosa Coss. & Balansa	No wing	Morocco	Morocco, Errachidia	T. Buira, J. Calvo and S. Hantson (MA 807960)	JX481145
Sect. *Diffusae*					
L. albifrons (Sibth. & Sm.) Steud.	No wing	SW Asia, S and E Mediterranean	Israel, Negev	A. Danin, S.G. Knees *et al.* (RNG)	JX481129
L. decipiens Batt.	No wing	Algeria	Algeria, l'Akfadou NP	A. Dubois (MA 589738)	JX481125

Table 3. Continued.

L. flava (Poir.) Desf.	No wing	Algeria, Corsica, Sardinia	Italy, Corsica	C. Bukanell and L. Ollum (E 00419551)	JX481130
L. haelava (Forssk) F.Dietr.	No wing	NE Africa, SW Asia	Israel, Horbat Medin	D. Heller and I. Shammash (MA 532177)	JX481122
L. hirta (L.) Moench	No wing	S Iberian Peninsula	Spain, Zamora	P. Bariego (MA 793918)	JX481120
L. joppensis Bornm.	No wing	Israel	Israel, Ashkeleton	A. Danin, S.G. Knees *et al.* (RNG)	JX481123
L. laxiflora Desf.	No wing	N Africa	Tunisia, Jerid, Cedada	C. Aedo *et al.* (MA 795183)	JX481128
L. reflexa (L.) Chaz.	No wing	N Africa	Algeria, Algiers	J. J. Aldasoro A9799 (MA)	JX481126
L. triphylla (L.) Mill.	No wing	W and S Mediterranean	Tunisia, El Vef	J. Calvo *et al.* (MA 797461)	JX481132
L. virgata (Poir.) Desf.	No wing	N Africa	Algeria, SE Constantine	D.A and S.J. Sutton (RNG)	JX481131
L. warionis Pomel	No wing	NW Africa	Morocco, Beni Tajjita	D. Podlech (MA 589733)	JX481127
Sect. *Supinae*					
Subsect. *Supinae*					
L. aeruginea (Gouan) Cav.	Wing	Iberian Peninsula	Spain, Granada	J.L. Blanco-Pastor 51JB09 (MA)	JQ814486
L. alpina (L.) Mill.	Wing	CS Europe	Spain, Huesca	S. Martín Bravo 571SMB05 (UPOS)	JQ814489
L. amethystea (Vent.) Hoffmanns. & Link	Wing	SW Europe, NW Africa	Spain, Ciudad Real	R. García Río (MA 712742)	JQ814490
L. amoi Campo ex Amo	Wing	S Spain	Spain, Malaga	J.L. Blanco-Pastor 37JB09 (MA)	JX481108
L. anticaria Boiss. & Reut.	Wing	S Spain	Spain, Málaga	J.L. Blanco-Pastor 33JB09 (MA)	JQ814491
L. arvensis (L.) Desf.	Wing	SWC Europe, NW Africa, SW Asia	Spain, Almería	S.L. Jury and R.N. Carter (RNG)	JQ814494
L. badalii Loscos	Wing	NW Spain	Spain, Leon	M.F. Gardner and S.G. Gardner (RNG)	JQ814495
L. bubanii Font Quer	Wing	NE Spain	Spain, Huesca	M. Carrasco (MA 609430)	JQ814537
L. caesia (Pers.) F.Dietr.	Wing	C Spain	Spain, Ciudad Real	A. Molina and J. Varela (RNG)	JX481105
L. depauperata Leresche ex Lange	Wing	SE Spain	Spain, Albacete	P.F. Cannon *et al.* (RNG)	JX481107
L. faucicola Leresche & Levier	Wing	NW Spain	Spain, Leon	F. Llamas *et al.* (MA 619920)	JX481117
L. filicaulis Boiss. ex Leresche & Levier	Wing	CN Spain	Spain, Leon	C.M. Romero Rodriguez (MA 789283)	JX481118
L. glacialis Boiss.	Wing	S Spain	Spain, Granada	J.L. Blanco-Pastor 43JB09 (MA)	JQ814504
L. glauca (L.) Chaz.	Wing	CE Spain	Spain, Madrid	J. Calvo (MA 790863)	JX481116
L. latifolia Desf.	Wing	SW Spain, NW Africa	Spain, Sevilla	Ladero and Rivas Goday (SEV 32442)	JX481124
L. lilacina Lange	Wing	SE Spain	Spain, Albacete	P. F. Cannon *et al.* (RNG)	JX481156
L. micrantha (Cav.) Hoffmanns. & Link	Wing	Mediterranean, SW Asia	Spain, Huelva	J.L. Blanco-Pastor 22JB09 (MA)	JQ814513
L. munbyana Boiss. & Reut.	Wing	W Mediterranean	Spain, Huelva	J.L. Blanco-Pastor 21JB09 (MA)	JQ814515
L. oblongifolia (Boiss.) Boiss. & Reut.	Wing	S Iberian Peninsula	Spain, Málaga	J.L. Blanco-Pastor 34JB09 (MA)	JQ814516
L. orbensis Carretero & Boira	Wing	E Iberian Peninsula	Spain, Alicante. Sagra	J.L. Blanco-Pastor 4JB10	JQ814518
L. platycalyx Boiss.	Wing	Spain	Spain, Cádiz	S. Martín Bravo 5SMB08 (UPOS)	JQ814520

Table 3. Continued.

L. polygalifolia Hoffmanns. & Link	Wing	Iberian Peninsula	Portugal, Monte Gordo	J.L. Blanco-Pastor 33JB10 (MA)	JQ814522
L. propinqua Boiss. & Reut.	Wing	N Spain	Spain, Castilla	Unknown collector (RNG)	JQ814524
L. saturejoides Boiss.	Wing	S Iberian Peninsula	Spain, Málaga	J.L. Blanco-Pastor 36JB09 (MA)	JQ814525
L. simplex Willd. ex Desf.	Wing	S Europe, N Africa, SW Asia	Greece, Ararchova	P. Vargas 79PV08 (MA)	JQ814528
L. supina (L.) Chaz.	Wing	SW Europe	France, Gorges de l'Hérault	J. Lambinon (RNG)	JQ814530
L. thymifolia (Vahl) DC.	Wing	SW France	France, Gironde	B. de Retz (MA 303566)	JX481106
L. tristis (L.) Mill.	Wing	S Spain NW Africa	Spain, Cádiz	P. Jiménez Mejías 105PJM04 (UPOS)	JX481109
L. verticillata Boiss.	Wing	S Spain	Spain, Granada	J.M. Losa (RNG)	JX481110
Subsect. *Saxatile*					
L. arenaria DC.	Marginal ridge	W France	France, Vendée	F. De Raeve (RNG)	JX481112
L. bipunctata (L.) Chaz.	Marginal ridge	NC Portugal	Spain, Soria	A. Segura (RNG)	JQ814496
L. coutinhoi Valdés	Marginal ridge	N Portugal	Portugal, Freixo-de-Espada	A. Teixeira s.n. (MA)	JX481113
L. diffusa Hoffmanns. & Link	Wing	NC Portugal	Portugal, Freixo-de-Espada	A. Teixeira s.n. (MA)	JX481114
L. huteri Lange	Marginal ridge	S Spain	Spain, Malaga	J.L. Blanco-Pastor 32JB09 (MA)	JX481111
L. oligantha Lange	Marginal ridge	SE Spain	Spain, Alicante	L. Serra (MA 753096)	JX481121
L. saxatilis (L.) Chaz.	Wing	NC Iberian Peninsula	Spain, Madrid	P. Vargas 20PV09 (MA)	JX481115
L. tursica Valdés & Cabezudo	No wing	SW Spain	Spain, Huelva	J.L. Blanco-Pastor 18JB09 (MA)	JQ814533
Sect. *Pelisserianae*					
L. pelisseriana (L.) Mill.	Wing	CE Mediterranean, W Europe	Turkey, Bayramiç	S. Castroviejo (MA 643850)	JX481082
L. triornithophora (L.) Willd.	Wing	W Iberian Peninsula	Spain, Cáceres, Sierra de Gata	M. Fernández-Mazuecos (18MF07)	JX481083
Sect. *Versicolores*					
Subsect. *Versicolores*					
L. algarviana Chav.	No wing	SW Portugal	Portugal, Cabo de São Vicente	M. Fernández-Mazuecos 11MF09 (MA)	JX481086
L. bipartita (Vent.) Willd.	No wing	W Morocco	Morocco, Rabat	S.L. Jury and R.G. Wilson 18558 (RNG)	JX481094
L. bordiana Santa & Simonneau	No wing	NW Africa	Algeria, Sidi Lakhdar	D.A. and S.J. Sutton 172 (RNG)	JX481095

Table 3. Continued.

L. clementei Haens.	No wing	S Spain	Spain, Málaga, Alhaurín de la Torre	M. Fernández-Mazuecos *et al.* 7MF08 (MA)	JX481089
L. gharbensis Batt. & Pit.	No wing	NW Africa	Spain, Huelva, Gibraleón	Fernández-Mazuecos *et al.* 7MF09 (MA)	JX481100
L. hellenica Turrill	No wing	Greece	Greece, Kambos	Unknown collector (ATH)	JX481102
L. incarnata (Vent.) Spreng.	No wing	SW Iberia, NW Africa	Spain, Salamanca, Pelabravo	M. Fernández-Mazuecos and P. Vargas 39MF09 (MA)	JX481088
L. maroccana Hook.f.	No wing	Morocco	Morocco, Marrakech – Tizi-n-Test	S.L. Jury *et al.* 14209 (RNG)	JX481097
L. multicaulis (L.) Mill.	No wing	N Africa, Sicilia	Italy, Sicily, Etna	I. Álvarez *et al.* IA1622 (MA)	JX481098
L. pedunculata (L.) Chaz.	No wing	W Mediterranean	Spain, Huelva, Marismas del Odiel	M. Fernández-Mazuecos *et al.* 4MF09 (MA)	JX481101
L. pseudoviscosa Murb.	No wing	Tunisia	Tunisia, El Haouaria	P. Wilkin and E.J. Wellens 231 (RNG)	JX481099
L. salzmannii Boiss.	No wing	S Spain	Spain, Málaga, El Chorro	M. Fernández-Mazuecos and J. Ramírez 19MF09 (MA)	JX481087
L. spartea (L.) Chaz.	No wing	SW Europe	Spain, Madrid, Colmenar	P. Vargas 101PV07 (MA)	JX481090
L. tenuis (Viv.) Spreng.	No wing	NC and NE Africa	Libya, Tripoli	Davis and Boulos 50581 (RNG)	JX481096
L. tingitana Boiss. & Reut.	No wing	NW Africa	Algeria, El Macta	D.A. and S.J. Sutton 383 (RNG)	JX481092
L. viscosa (L.) Chaz.	No wing	S and W Iberia	Spain, Huelva, Marismas del Odiel	M. Fernández-Mazuecos *et al.* 6MF09 (MA)	JX481091
L. weilleri Emb. & Maire	No wing	S Morocco	Morocco, Tirhmi	Miller, Russell and Sutton s.n. (RNG)	JX481093
Subsect. *Elegantes*					
L. elegans Cav.	No wing	W Iberia	Portugal, Manteigas	M. Fernández-Mazuecos 127MF10 (MA)	JX481103
L. nigricans Lange	No wing	SE Spain	Spain, Almería, Tabernas	P. Vargas 3PV08 (MA)	JX481104
Sect. *Macrocentrum*					
L. armeniaca Chav.	No wing	SW Asia	Armenia, Gegharkunik	C. Aedo *et al.* (MA 743447)	JX481080
L. chalepensis (L.) Mill.	No wing	NC and E Mediterranean	Cyprus, Cape Kiti	Iter Mediterraneum IV (MA 495681)	JX481081
Nuttallanthus					
N. canadensis (L.) D.A.Sutton [= *L. canadensis* (L.) Dum.Cours.]	No wing	USA, Canada	USA, Alabama, Pike County	C.E. Freeman (UTEP 65885)	AY883085
N. subandinus (Diels) D.A.Sutton [= *L. subandina* Diels]	No wing	S America	Brazil, Sao Francisco de Paula	Grazziotin and Perazzolo (MA 406417)	JX481084
N. texanus (Scheele) D.A.Sutton [= *L. texana* Scheele]	No wing	USA, Mexico	USA, Del Mar Mesa	D.E. Breedlove (MA 494665)	JX481085

amplified the internal transcribed spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA using primer combinations 17SE-26SE (Sun *et al.*, 1994) or ITS5-ITS4 (White *et al.*, 1990; Sang *et al.*, 1995). PCR reactions consisted of 1 min pretreatment at 94ºC and 30 cycles of 1 min at 94ºC, 1 min at 50-54ºC and 1 min at 72ºC. In some cases, it was needed to amplify the ITS1 and ITS2 regions separately using internal primers designed in the conserved 5.8S region. All amplified products were submitted to Macrogen Inc. (Seoul, South Korea) for sequencing using primers ITS5 and ITS4. Resulting sequence data were assembled and edited in Geneious Pro v5 (Drummond *et al.*, 2010), and submitted to GenBank (see Table 3 for accession numbers). Sequences of one additional species of *Nuttallanthus* (*N. canadensis*) and 71 species representing the remaining 26 genera of Antirrhineae recognized by Sutton (1988) and Vargas (2004) were retrieved from the GenBank database (Oyama & Baum, 2004; C.E. Freeman, unpublished data; Vargas *et al.*, 2004; Vargas *et al.*, 2009), except for the sequence of *Maurandya scandens*, which was newly generated. We also retrieved from GenBank the ITS sequence of *Lafuentea rotundifolia*, which is closely related to Antirrhineae (Albach *et al.*, 2005), and seven additional species representing as many Lamiales genera to be used as the outgroup following Vargas *et al.* (2004) (see Supporting Information Table S1).

Phylogenetic analysis

All sequences were aligned using MAFFT 6 (Katoh *et al.*, 2002) with default parameters, and further adjustments were made by visual inspection. Two different datasets were prepared: (i) the Antirrhineae dataset, constituted by all 168 sampled sequences of Antirrhineae (including the 94 sequences of *Linaria*, three of *Nuttallanthus* and 71 of other genera) plus the eight additional sequences of Lamiales; and (ii) the *Linaria* dataset, including the 94 sequences of *Linaria* and three of *Nuttallanthus*, plus *Schweinfurthia latifolia* and *Galvezia fruticosa*. The *Linaria* dataset was prepared to gain resolution within the *Linaria* lineage (see below), and in order to employ this analysis for hypothesis testing. Phylogenetic analyses were conducted on both datasets using Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP). *Halleria lucida* was used as the outgroup for the Antirrhineae dataset (as in Vargas *et al.*, 2004), and *Galvezia fruticosa* for the *Linaria* dataset (based on the analysis of the Antirrhineae dataset, see below). For ML and BI analyses, the best-fitting substitution model (GTR+G for

both datasets) was determined under the Akaike Information Criterion (AIC) in jModelTest 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). BI was performed in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) using two runs with 20 million generations each and a sample frequency of 1000. Chain convergence was assessed with Tracer 1.4 (Rambaut & Drummond, 2007). A 50% majority-rule consensus tree with Bayesian posterior probabilities (PP) of clades was calculated after removing the first 10% generations as burn-in. ML was implemented in PhyML 3.0 (Guindon & Gascuel, 2003; Guindon *et al.*, 2010) using the Nearest Neighbor Interchange branch-swapping algorithm. One-thousand non-parametric bootstrap replicates (ML-BS) were applied. MP analyses were performed in TNT 1.1 (Goloboff *et al.*, 2003) using a heuristic search with 10,000 replicates saving two most-parsimonious trees per replicate, followed by a second heuristic search retaining all best trees and using the trees obtained in the previous 10,000 replicates as the starting ones. Bootstrap support (MP-BS) of clades was assessed with 10,000 standard replicates.

Bayesian hypothesis testing

Bayes factors (BF) allow testing of alternative hypotheses in a Bayesian framework (Kass & Raftery, 1995; Suchard *et al.*, 2001). They quantify the support for one hypothesis *versus* another given the data. We used this approach, implemented in Tracer 1.4 (Rambaut & Drummond, 2007), to test eight alternative phylogenetic and systematic hypotheses (constrained tree topologies) *versus* an unconstrained analysis H_0 (corresponding to the Bayesian analysis of the *Linaria* dataset, as described above). Monophyly constraints involving several taxonomical entities (Valdés, 1970; Viano, 1978b; Sutton, 1988) were set in each MrBayes analysis: H_1 – monophyly of genus *Linaria* (excluding American species); H_2 – monophyly of sect. *Linaria*; H_3 – monophyly of sect. *Diffusae*; H_4 – monophyly of sect. *Speciosae*; H_5 – monophyly of sect. *Supinae*; H_6 – monophyly of subsect. *Supinae*; H_7 – monophyly of subsect. *Saxatile*; H_8 – monophyly of winged species and wingless species. Stationarity and convergence of analyses were assessed in Tracer after discarding the first 10% of sampled generations as burn-in. Marginal likelihoods, their standard errors (estimated using 1000 bootstrap replicates) and BFs were calculated. We considered 2xlnBF(H *vs.* H_0) -2 to -6 as positive evidence against H in favor of H_0; 2xlnBF(H

vs. H_0) -6 to -10 as strong evidence against *H* in favor of H_0; and 2xlnBF(*H vs.* H_0) < -10 as very strong evidence against *H* in favor of H_0 (Kass & Raftery, 1995).

Reconstruction of seed trait evolution

To analyze the evolution of seed traits (seed wing), we reconstructed ancestral states using the "trace character over trees" analysis implemented in Mesquite 2.75 (Maddison & Maddison, 2011). Based on available micromorphological studies of *Linaria* seeds (Sutton, 1988), three character states were defined: (1) no wing; (2) wing (0.1-1.2 mm broad); and (3) marginal ridge (0.01-0.1 mm broad) (Table 3; Fig. 2). All trees from the stable posterior distribution of the Bayesian analysis of the *Linaria* dataset were used to account for the uncertainty in tree topology. Analyses were performed using both parsimony and likelihood reconstruction methods. For the likelihood reconstruction we used the one-parameter Markov k-state model (Mk1) (Lewis, 2001) with pre-defined parameters, thus assigning equal probability to any type of shift. Additionally, the "summarize state changes over trees" option was used to summarize the number of changes between character states across the Bayesian posterior distribution of trees.

Results

Phylogenetic analysis

The Antirrhineae dataset had a total aligned length of 675 bp with 300 parsimony-informative characters, while the *Linaria* dataset had 621 bp with 183 parsimony-informative characters (Table 4). Overall, the BI, ML and MP analyses yielded congruent topologies, except for some weakly supported clades (Figs. 3 and 4). Lower support values were generally obtained in the MP analysis than in the ML and BI analyses.

Monophyly of Antirrhineae was strongly supported in the BI analysis (PP = 1) of the Antirrhineae dataset (Fig. 3), with *Lafuentea* as sister taxon (PP = 1). Six major well-supported clades were recognized within Antirrhineae, which have been given the name of one representative genus:

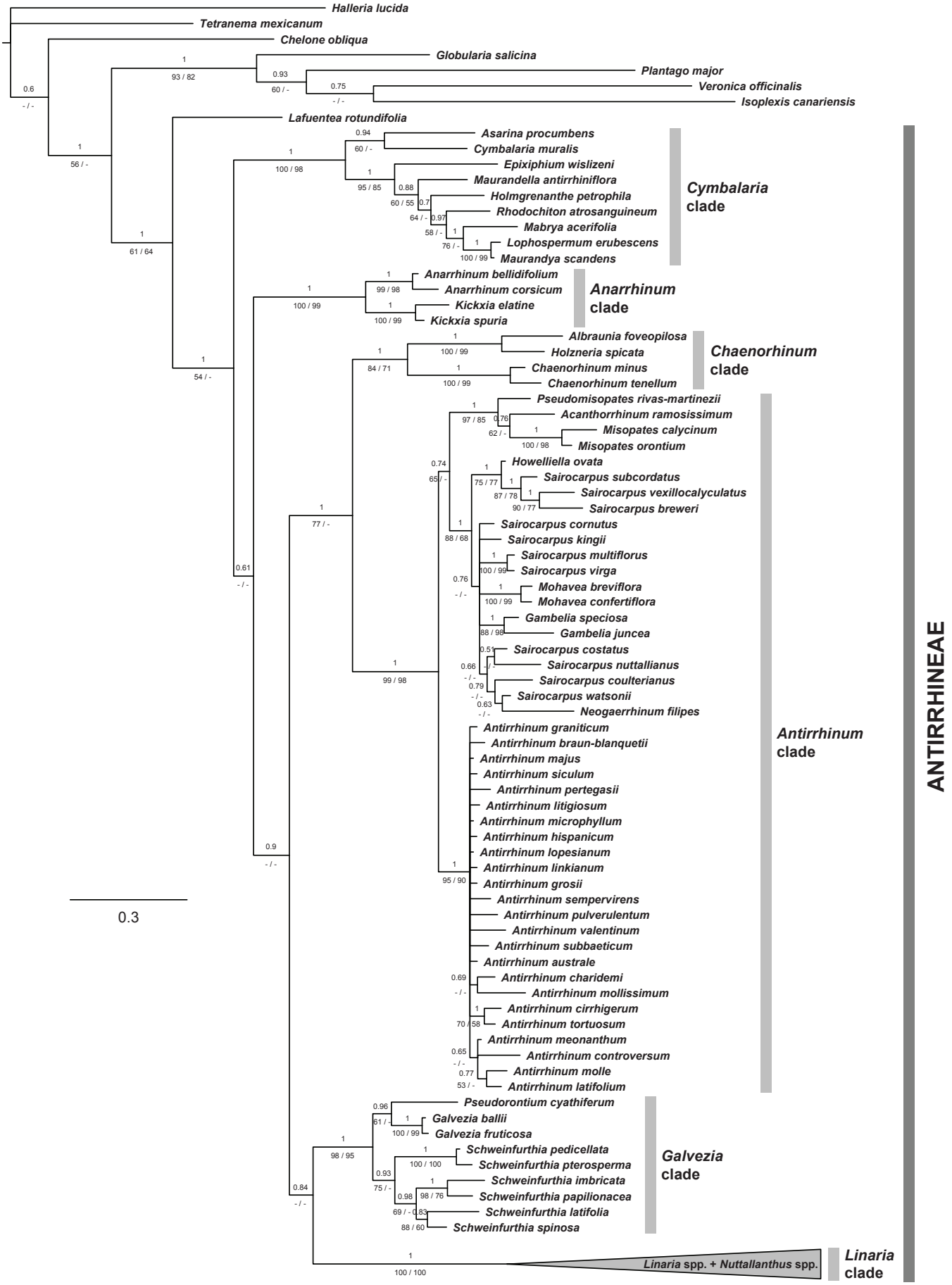
Table 4. Characteristics of both datasets of ITS sequences employed in phylogenetic analyses.

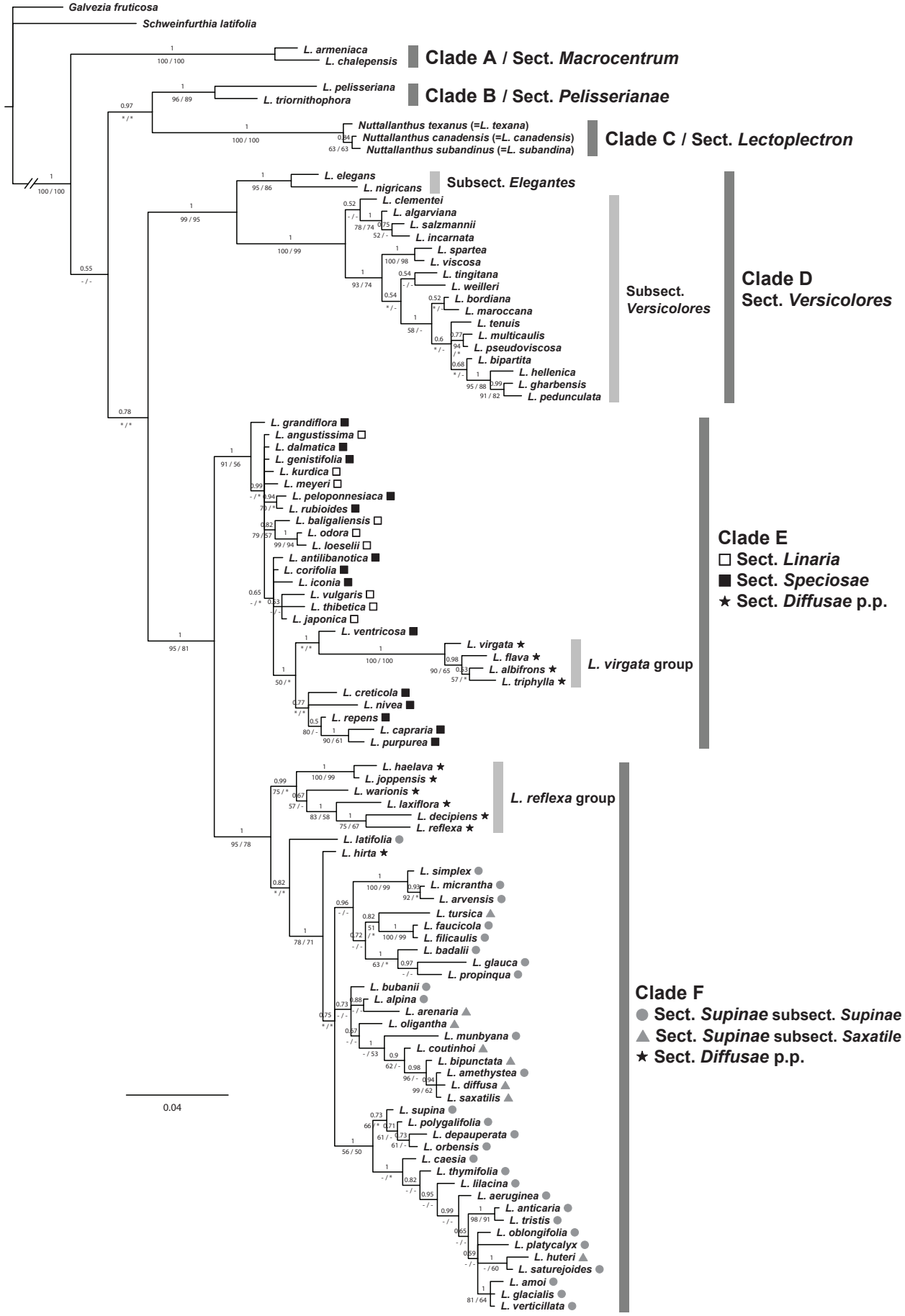
	Antirrhineae dataset	***Linaria* dataset**
Sequences	176	99
Aligned length (bp)	675	621
Ungapped length range	550-623	584-600
Pairwise % identity	82.5	90.2
Variable characters	397	237
Parsimony-informative characters	300	183
Mean % G+C content	59.6	58.6
Substitution model	GTR+G	GTR+G

the *Cymbalaria* clade (9 genera; PP = 1; ML-BS = 100%; MP-BS = 98%); the *Anarrhinum* clade (2 genera; PP = 1; ML-BS = 100%; MP-BS = 99%); the *Chaenorhinum* clade (3 genera; PP = 1; ML-BS = 84%; MP-BS = 71%); the *Antirrhinum* clade (9 genera; PP = 1; ML-BS = 99%; MP-BS = 98%); the *Galvezia* clade (3 genera; PP = 1; ML-BS = 98%; MP-BS = 95%); and the *Linaria* clade, constituted by all sampled species of *Linaria* and *Nuttallanthus* (PP = 1; ML-BS = 100%; MP-BS = 100%). Relationships among these clades were weakly supported, except for the sister-group relationship between the *Chaenorhinum* and *Antirrhinum* clades, which was supported by BI (PP = 1) and ML (ML-BS = 77%).

Within the *Linaria* clade, analyses of both datasets yielded congruent phylogenetic relationships. Therefore, only results of the *Linaria* dataset (Fig. 4) are shown and discussed at this level. Six major clades (A-F) were recognized by the three phylogenetic analyses. Clades A, B and C were respectively formed by the two species of sect. *Macrocentrum* (PP = 1; ML-BS = 100%; MP-BS = 100%), the two species of sect. *Pelisserianae* (PP = 1; ML-BS = 96%; MP-BS = 89%) and

Fig. 3. Phylogenetic analysis of the Antirrhineae dataset (168 ITS sequences of Antirrhineae, including 94 of *Linaria* and 3 of *Nuttallanthus*). The fifty-percent majority-rule consensus tree obtained in the Bayesian analysis is shown. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood / maximum parsimony percentage bootstrap values. An asterisk (*) indicates no bootstrap support over 50% but clade present in the maximum likelihood tree / strict consensus tree of the maximum parsimony analysis. A hyphen (-) indicates no bootstrap support over 50% and clade absent from the maximum likelihood tree / strict consensus tree of the maximum parsimony analysis. The *Linaria* clade has been collapsed for clarity (see Fig. 4).


Halleria lucida
Tetranema mexicanum
Chelone obliqua
Globularia salicina
Plantago major
Veronica officinalis
Isoplexis canariensis
Lafuentea rotundifolia
Asarina procumbens
Cymbalaria muralis
Epixiphium wislizeni
Maurandella antirrhiniflora
Holmgrenanthe petrophila
Rhodochiton atrosanguineum
Mabrya acerifolia
Lophospermum erubescens
Maurandya scandens
Cymbalaria clade
Anarrhinum bellidifolium
Anarrhinum corsicum
Kickxia elatine
Kickxia spuria
Anarrhinum clade
Albraunia foveopilosa
Holzneria spicata
Chaenorhinum minus
Chaenorhinum tenellum
Chaenorhinum clade
Pseudomisopates rivas-martinezii
Acanthorrhinum ramosissimum
Misopates calycinum
Misopates orontium
Howelliella ovata
Sairocarpus subcordatus
Sairocarpus vexillocalyculatus
Sairocarpus breweri
Sairocarpus cornutus
Sairocarpus kingii
Sairocarpus multiflorus
Sairocarpus virga
Mohavea breviflora
Mohavea confertiflora
Gambelia speciosa
Gambelia juncea
Sairocarpus costatus
Sairocarpus nuttallianus
Sairocarpus coulterianus
Sairocarpus watsonii
Neogaerrhinum filipes
Antirrhinum graniticum
Antirrhinum braun-blanquetii
Antirrhinum majus
Antirrhinum siculum
Antirrhinum pertegasii
Antirrhinum litigiosum
Antirrhinum microphyllum
Antirrhinum hispanicum
Antirrhinum lopesianum
Antirrhinum linkianum
Antirrhinum grosii
Antirrhinum sempervirens
Antirrhinum pulverulentum
Antirrhinum valentinum
Antirrhinum subbaeticum
Antirrhinum australe
Antirrhinum charidemi
Antirrhinum mollissimum
Antirrhinum cirrhigerum
Antirrhinum tortuosum
Antirrhinum meonanthum
Antirrhinum controversum
Antirrhinum molle
Antirrhinum latifolium
Antirrhinum clade
Pseudorontium cyathiferum
Galvezia ballii
Galvezia fruticosa
Schweinfurthia pedicellata
Schweinfurthia pterosperma
Schweinfurthia imbricata
Schweinfurthia papilionacea
Schweinfurthia latifolia
Schweinfurthia spinosa
Galvezia clade
Linaria spp. + Nuttallanthus spp.
Linaria clade
ANTIRRHINEAE
0.3


Galvezia fruticosa
Schweinfurthia latifolia
L. armeniaca
L. chalepensis
Clade A / Sect. Macrocentrum
L. pelisseriana
L. triornithophora
Clade B / Sect. Pelisserianae
Nuttallanthus texanus (=L. texana)
Nuttallanthus canadensis (=L. canadensis)
Nuttallanthus subandinus (=L. subandina)
Clade C / Sect. Lectoplectron
L. elegans
L. nigricans
Subsect. Elegantes
L. clementei
L. algarviana
L. salzmannii
L. incarnata
L. spartea
L. viscosa
L. tingitana
L. weilleri
L. bordiana
L. maroccana
L. tenuis
L. multicaulis
L. pseudoviscosa
L. bipartita
L. hellenica
L. gharbensis
L. pedunculata
Subsect. Versicolores
Clade D
Sect. Versicolores
L. grandiflora
L. angustissima
L. dalmatica
L. genistifolia
L. kurdica
L. meyeri
L. peloponnesiaca
L. rubioides
L. baligaliensis
L. odora
L. loeselii
L. antilibanotica
L. corifolia
L. iconia
L. vulgaris
L. thibetica
L. japonica
L. ventricosa
L. virgata ★
L. flava ★
L. albifrons ★
L. triphylla ★
L. virgata group
L. creticola
L. nivea
L. repens
L. capraria
L. purpurea
Clade E
□ Sect. Linaria
■ Sect. Speciosae
★ Sect. Diffusae p.p.
L. haelava ★
L. joppensis ★
L. warionis ★
L. laxiflora ★
L. decipiens ★
L. reflexa ★
L. reflexa group
L. latifolia ●
L. hirta ★
L. simplex ●
L. micrantha ●
L. arvensis ●
L. tursica ▲
L. faucicola ●
L. filicaulis ●
L. badalii ●
L. glauca ●
L. propinqua ●
L. bubanii ●
L. alpina ●
L. arenaria ▲
L. oligantha ▲
L. munbyana ●
L. coutinhoi ▲
L. bipunctata ▲
L. amethystea ●
L. diffusa ▲
L. saxatilis ▲
L. supina ●
L. polygalifolia ●
L. depauperata ●
L. orbensis ●
L. caesia ●
L. thymifolia ●
L. lilacina ●
L. aeruginea ●
L. anticaria ●
L. tristis ●
L. oblongifolia ●
L. platycalyx ●
L. huteri ▲
L. saturejoides ●
L. amoi ●
L. glacialis ●
L. verticillata ●
Clade F
● Sect. Supinae subsect. Supinae
▲ Sect. Supinae subsect. Saxatile
★ Sect. Diffusae p.p.
0.04

the three sampled species of *Nuttallanthus* (PP = 1; ML-BS = 100%; MP-BS = 100%). All 19 sampled species of sect. *Versicolores* constituted clade D (PP = 1; ML-BS = 99%; MP-BS = 95%). Within clade D, subsections *Elegantes* (PP = 1; ML-BS = 95%; MP-BS = 86%) and *Versicolores* (PP = 1; ML-BS = 100%; MP-BS = 99%) formed monophyletic lineages that were sister to each other. Clade E (PP = 1; ML-BS = 91%; MP-BS = 56%) was formed by all sampled species of sect. *Linaria*, all sampled species of sect. *Speciosae* and four species of sect. *Diffusae*. The latter four taxa formed a well-supported monophyletic lineage (PP = 1; ML-BS = 100%; MP-BS = 100%). Finally, clade F (PP = 1; ML-BS = 95%; MP-BS = 78%) was formed by the remaining species of sect. *Diffusae* and all sampled species of sect. *Supinae*.

Relationships between major clades were poorly resolved, except for the sister-group relationship between clades E and F (PP = 1; ML-BS = 95%; MP-BS = 81%). A sister-group relationship between clades B and C was supported by BI (PP = 1). This relationship lacked statistical support in the MP and ML analyses, although a B-C clade was recovered in the strict consensus tree of the MP analysis and in the ML tree. On the other hand, relationships between clades A, B+C, D and E+F remained unsupported in all three analyses.

Bayesian hypothesis testing

The Bayes Factor analysis (Table 5), recovered decisive (very strong) support (2xlnBF < -10) for rejection of five out of eight phylogenetic hypotheses: H_3 (monophyly of sect. *Diffusae*); H_4 (monophyly of sect. *Speciosae*); H_5 (monophyly of sect. *Supinae*); H_6 (monophyly of subsect.

Fig. 4. Phylogenetic analysis of the *Linaria* dataset (94 ITS sequences of *Linaria* and 3 of *Nuttallanthus*). The fifty-percent majority-rule consensus tree obtained in the Bayesian analysis is shown. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood / maximum parsimony percentage bootstrap values. An asterisk (*) indicates no bootstrap support over 50% but clade present in the maximum likelihood tree / strict consensus tree of the maximum parsimony analysis. A hyphen (-) indicates no bootstrap support over 50% and clade absent from the maximum likelihood tree / strict consensus tree of the maximum parsimony analysis. Delimitation of sections and subsections follows Sutton (1988), except for sect. *Lectoplectron*, which follows Valdés (1970).

Table 5. Mean values of marginal likelihood (with standard error of mean) and Bayes factor test statistics (2xlnBF) for the unconstrained analysis (H_0) and the tested phylogenetic hypotheses (H_1-H_8). 2xlnBF(H_n *vs.* H_0) -2 to -6 reflects positive evidence against H_n in favor of H_0; 2xlnBF(H_n *vs.* H_0) -6 to -10 reflects strong evidence against H_n in favor of H_0 and 2xlnBF(H *vs.* H_0) < -10 reflects very strong evidence against H_n in favor of H_0 (Kass & Raftery, 1995).

Hypothesis (H)	Marginal likelihood (lnP(model | data)) ± SE	2xlnBF(H_n *vs.* H_0)
H_0: unconstrained analysis	-5348.930 ± 0.367	-
H_1: monophyly of genus *Linaria*	-5352.833 ± 0.281	-7.808
H_2: monophyly of sect. *Linaria*	-5351.374 ± 0.310	-4.888
H_3: monophyly of sect. *Diffusae*	-5398.932 ± 0.336	-100.006
H_4: monophyly of sect. *Speciosae*	-5564.360 ± 0.348	-430.862
H_5: monophyly of sect. *Supinae*	-5358.284 ± 0.293	-18.71
H_6: monophyly of subsect. *Supinae*	-5466.603 ± 0.325	-235.348
H_7: monophyly of subsect. *Saxatile*	-5399.977 ± 0.327	-102.096
H_8: monophyly of winged species and wingless species	-5591.133 ± 0.351	-484.406

Supinae); H_7 (monophyly of subsect. *Saxatile*) and H_8 (monophyly of winged species and wingless species). Hypotheses H_2 (monophyly of sect. *Linaria*) and H_1 (monophyly of genus *Linaria*) were not decisively but positively (2xlnBF -2 to -6) and strongly (and 2xlnBF -6 to -10) rejected respectively.

Reconstruction of seed trait evolution

Ancestral state reconstructions revealed the seed wing as a character that has changed several times in the course of *Linaria* evolution (Figs. 5 and 6). The maximum likelihood reconstruction (Fig. 5) and the parsimony reconstruction (not shown) yielded congruent results, although with higher resolution at the common ancestor of the *Linaria* clade in the parsimony analysis, which was reconstructed as having wingless seeds (100% of trees). In the likelihood reconstruction, the ancestral state for the common ancestor of the *Linaria* clade remained uncertain, but the wingless state was reconstructed as ancestral for five of the six major clades with variable support: clades A (100% of trees), C (100%), D (100%), E (84%) and F (60%). On the other hand, winged seeds were recovered as ancestral for clade B (78%). Therefore, the presence

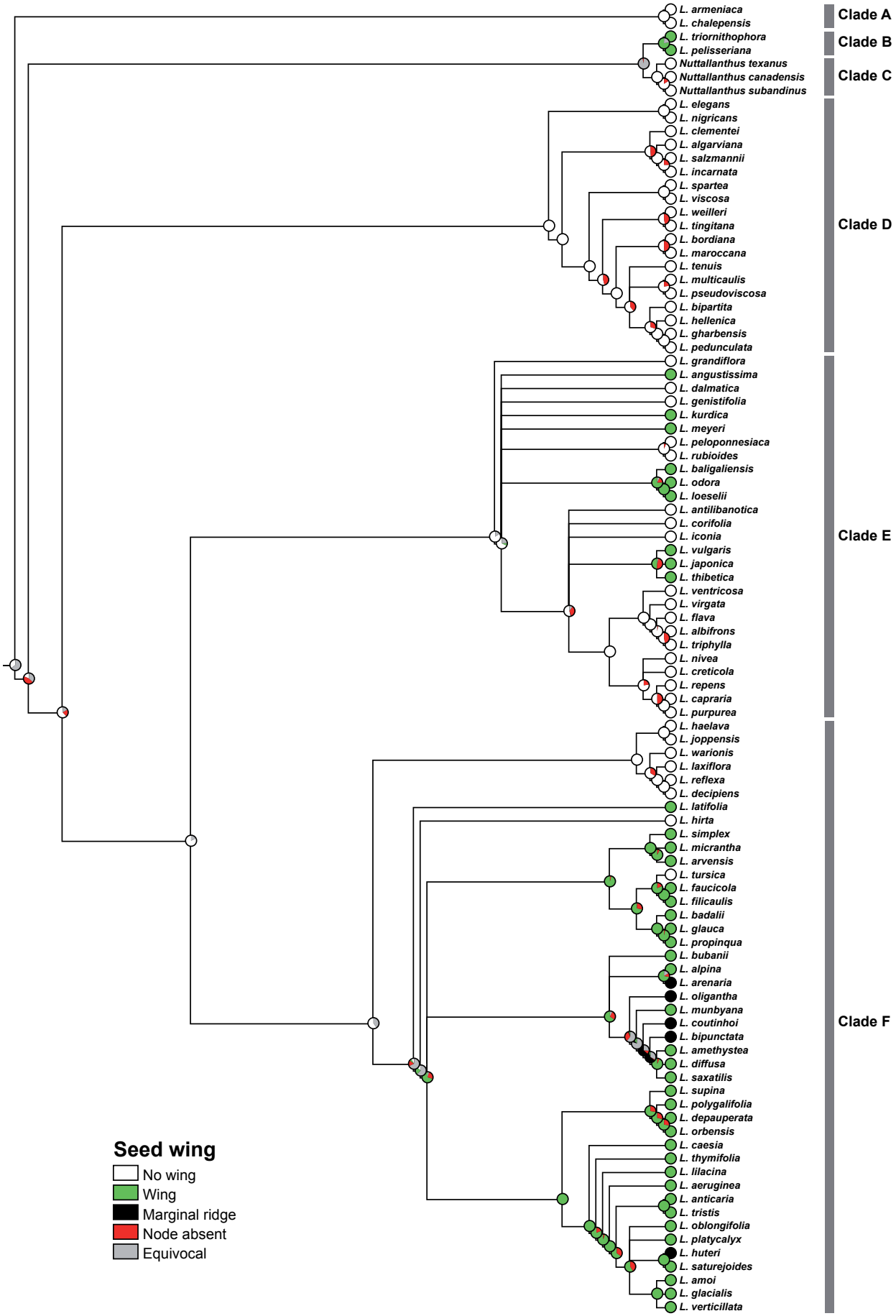
of winged seeds in clades B (sect. *Pelisserianae*), E (sect. *Linaria*) and F (sect. *Supinae*) was attributable to at least three independent origins. Low resolution in clade E prevented from reliable reconstruction within this group, although more than one shift from wingless to winged seeds may have occurred. In clade F, it remained unclear whether two shifts from wingless to winged seeds have occurred (one in *L. latifolia* and the other in the ancestor of the remaining *Supinae* species) or one shift with one posterior lost of wing in *L. hirta*. In the same clade, one lost of wing was clearly detected in *L. tursica*, along with several reductions from wing to marginal ridge (in *L. arenaria*, *L. oligantha-L. coutinhoi-L bipunctata* and *L. huteri,* see Fig. 5).

When summarizing the number of changes across the posterior distribution of trees using likelihood optimization (Fig. 6), we obtained high uncertainty on the number of changes from wingless to winged seeds. Nevertheless, 87% of trees yielded one or more well-supported events, with an average of 2.32. No changes were reconstructed from no wing to marginal ridge and from marginal ridge to no wing, while we obtained one or more changes from wing to no wing (average 1.68) and from wing to marginal ridge (average 2.19). Finally, we obtained zero to two changes from marginal ridge to wing (average 0.67).

Discussion

Our deep sampling of *Linaria* and *Nuttallanthus*, as well as the addition of five genera (*Mabrya*, *Maurandya*, *Holmgrenanthe*, *Epixiphium* and *Galvezia*) to previously analyzed ITS sequences (Oyama & Baum, 2004; Vargas *et al.*, 2004; Vargas *et al.*, 2009) make this the most deeply sampled phylogenetic hypothesis of the Antirrhineae tribe published to date (Figs. 3 and 4). Phylogenetic relationships among Antirrhineae genera herein reported are congruent with those obtained by Vargas *et al.* (2004) based on the same DNA region. Therefore, we are for

Fig. 5. Evolution of seed wing in the *Linaria* clade. The tree is the fifty-percent majority-rule consensus from the Bayesian analysis. Pie charts at nodes summarize the results of the likelihood character optimization in Mesquite, conducted over the posterior distribution of trees from the Bayesian analysis. Each chart shows the proportion of trees for which a given seed wing state was reconstructed for that node.


L. armeniaca
L. chalepensis
L. triornithophora
L. pelisseriana
Nuttallanthus texanus
Nuttallanthus canadensis
Nuttallanthus subandinus
L. elegans
L. nigricans
L. clementei
L. algarviana
L. salzmannii
L. incarnata
L. spartea
L. viscosa
L. weilleri
L. tingitana
L. bordiana
L. maroccana
L. tenuis
L. multicaulis
L. pseudoviscosa
L. bipartita
L. hellenica
L. gharbensis
L. pedunculata
L. grandiflora
L. angustissima
L. dalmatica
L. genistifolia
L. kurdica
L. meyeri
L. peloponnesiaca
L. rubioides
L. baligaliensis
L. odora
L. loeselii
L. antilibanotica
L. corifolia
L. iconia
L. vulgaris
L. japonica
L. thibetica
L. ventricosa
L. virgata
L. flava
L. albifrons
L. triphylla
L. nivea
L. creticola
L. repens
L. capraria
L. purpurea
L. haelava
L. joppensis
L. warionis
L. laxiflora
L. reflexa
L. decipiens
L. latifolia
L. hirta
L. simplex
L. micrantha
L. arvensis
L. tursica
L. faucicola
L. filicaulis
L. badalii
L. glauca
L. propinqua
L. bubanii
L. alpina
L. arenaria
L. oligantha
L. munbyana
L. coutinhoi
L. bipunctata
L. amethystea
L. diffusa
L. saxatilis
L. supina
L. polygalifolia
L. depauperata
L. orbensis
L. caesia
L. thymifolia
L. lilacina
L. aeruginea
L. anticaria
L. tristis
L. oblongifolia
L. platycalyx
L. huteri
L. saturejoides
L. amoi
L. glacialis
L. verticillata
Clade A
Clade B
Clade C
Clade D
Clade E
Clade F
Seed wing
No wing
Wing
Marginal ridge
Node absent
Equivocal

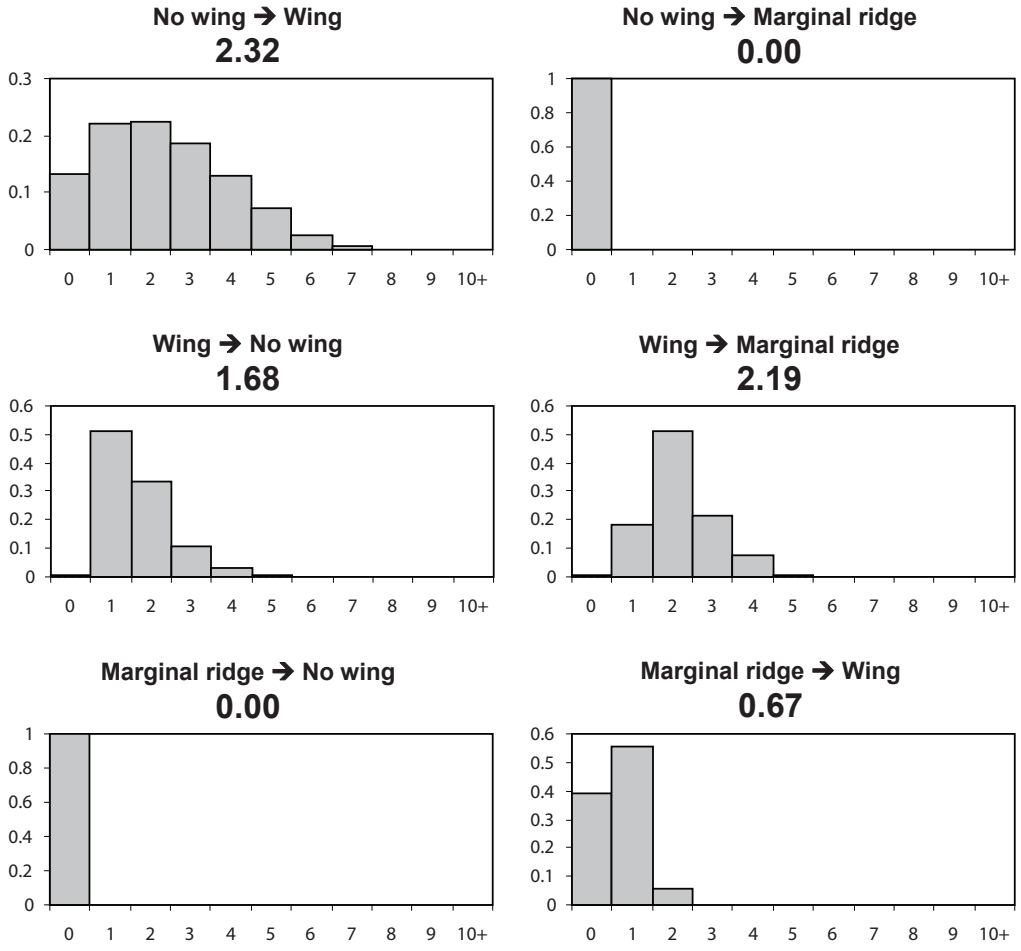


Fig. 6. Summary distributions of the number of changes between character states of seed wing, inferred when implementing likelihood optimization over the posterior distribution of trees from the Bayesian analysis of the *Linaria* clade. The average number of changes for each particular transition is shown above the histograms.

the first time able to place all Antirrhineae genera within the six major clades of Antirrhineae previously recognized (Fig. 3) (Vargas *et al.*, 2004), and to assess the phylogenetic relationships of *Linaria* and *Nuttallanthus* in a wide evolutionary framework.

Naturalness of Linaria including New World species

Naturalness of *Linaria* was first suggested by Vargas *et al.* (2004) based on a strongly supported monophyletic group encompassing eight sampled species of *Linaria* representing the seven

sections of Sutton's classification. This analysis did not, however, include any sample of the American genus *Nuttallanthus*. In our ITS phylogeny including 94 species of *Linaria*, this genus, as circumscribed by Sutton (1988), was recovered as a paraphyletic group, with the three sampled species of *Nuttallanthus* (*N. texanus*, *N. subandinus* and the type species *N. canadensis*) nested within it (clade C, Fig. 4). Furthermore, Bayes factors (Table 5) provided strong evidence against the monophyly of *Linaria* excluding *Nuttallanthus*.

North American spurred species of Antirrhineae were classically included within *Linaria* as *L. canadensis* (Chavannes, 1833; Bentham, 1846; Wettstein, 1895). Pennell (1935) recognized three North American species of *Linaria*: *L. canadensis*, *L. texana* and *L. floridana*, while a South American species (*L. subandina*) was first described by Diels (1906). Sutton (1988) argued that separation of these four American species as a distinct genus was justified on the basis of several morphological traits of flowers and seeds: the abaxial lip of the corolla greatly exceeding the adaxial lip; the weakly-developed palate that barely occludes the corolla tube; the very slender or absent spur; and prismatic seeds with four to seven ridges. However, we need to stress that (1) flower morphology of *Nuttallanthus* species is noticeably similar to that found in the two species of *Linaria* sect. *Macrocentrum* and some species of sect. *Versicolores* (compare, for example, the poorly developed palates and slender spurs of *L. nigricans*, *L. armeniaca* and *N. texanus* in Figs. 1V, 1Y and 1Z); and (2) seed morphology is highly variable among *Linaria* sections (Sutton, 1988). Therefore, separation of New World toadflax species at generic level is hardly justified on morphological and phylogenetic (Fig. 4) grounds. The fact that the three species constitute a well-supported monophyletic group within the *Linaria* clade, together with the distinct seed morphology, supports the circumscription of American species in a separate section of *Linaria*. This approach was first proposed by Pennell (1919, 1935), who treated the group as sect. *Lectoplectron*, and was later supported by Valdés (1970). However, this view has been abandoned in recent times in favor of Sutton's approach (e.g. Crawford & Elisens, 2006). Sutton's separation of New World Antirrhineae as distinct genera from those of the Old World has been generally supported by molecular phylogenies (Ghebrehiwet *et al.*, 2000; Vargas *et al.*, 2004). *Nuttallanthus* seems to be an exception, and we suggest that new accounts should treat American toadflax species as *Linaria* in order to preserve the naturalness of the genus. Indeed, monophyly of *Linaria* (including *Nuttallanthus*) is not only supported by ITS sequences, but also by the basic chromosome number x = 6 and a set of morphological traits that are not found

together elsewhere in Antirrhineae: presence of hypocotylary stems; entire, sessile, pinnately veined leaves; terminal, bracteate, racemose inflorescences; and spurred flowers (Valdés, 1970; Sutton, 1988). Nevertheless, further analyses with additional DNA regions will be of interest to support the circumscription of New World toadflaxes in a section within *Linaria*, as supported by the present ITS phylogeny.

Naturalness of infrageneric classification of Linaria

When comparing the main lineages of our phylogeny with recent infrageneric classifications of *Linaria* (Fig. 4; Table 2), it is apparent that some sections and subsections that are well-defined by distinct morphological traits were also found to be monophyletic. This is the case of the sections *Macrocentrum*, *Pelisserianae* and *Versicolores*.

Sect. *Macrocentrum*

The two species of sect. *Macrocentrum* (*L. chalepensis* and *L. armeniaca*, clade A; Figs. 1X and 1Y) display some unusual traits that are unique or rare within *Linaria* and which led Sutton (1980) to separate them from sect. *Versicolores* (where they had been included before; Bentham, 1846). First of all, in *L. chalepensis* and *L. armeniaca* the adaxial lobe of the calyx is shorter than the remaining four abaxial lobes. This trait is not found elsewhere in *Linaria*, but also occurs in *Holzneria*, which is a genus not closely related to *Linaria* according to nuclear (Vargas *et al.*, 2004; Fig. 3) and plastid (Appendix 1) phylogenies. Second, the small, lateral appendage present at the base of each stamen filament is considered unique within Antirrhineae (Sutton, 1980). And third, seeds have five or six longitudinal angles, a trait shared with *Nuttallanthus*, but not with the other sections of *Linaria*.

Sect. *Pelisserianae*

This section is constituted by two species (*L. triornithophora* and *L. pelisseriana*, clade B; Figs. 1Q and 1R) that were recovered as sister to each other, and in turn constituted the sister group

to *Nuttallanthus* according to the Bayesian analysis. *L. triornithophora* and *L. pelisseriana* are rather different in terms of habit, as well as disposition, size and shape of flowers and leaves. The two species had been respectively placed in sections *Grandes* (= sect. *Linaria*) and *Arvenses* (Bentham, 1846; Wettstein, 1895) until Valdés (1970) reunited them on the basis of capsule morphology and structure. While the discoid seeds of other winged-seeded species of *Linaria* are laterally compressed, in *L. triornithophora* and *L. pelisseriana*, seeds are dorsi-ventrally compressed (Sutton, 1988). This peculiar pattern of seed symmetry of *L. triornithophora* and *L. pelisseriana* is not found elsewhere in the genus, and appears to be a synapomorphy of the sect. *Pelisserianae*.

Sect. *Versicolores*

The morphological distinctness of the section *Versicolores*, as defined by Sutton (1988) (clade D; Figs. 1S-1W), was considered to be based on a diagnostic morphological synapomorphy not found elsewhere in the genus or even the tribe: a divided style with discrete stigmatic areas. A bifid style is clearly observed in the majority of species, while a merely emarginated stigma is found in *L. elegans* and *L. nigricans*. Viano (1978a, b) recognized both groups as independent sections (*Versicolores* and *Elegantes* respectively) on the basis of stigma and seed morphology, while Sutton (1988) considered them as subsections within the sect. *Versicolores*. The two groups were revealed as monophyletic and sister to each other in our ITS phylogeny, which is in agreement with cpDNA-based results (Fernández-Mazuecos & Vargas, 2011; Chapter 3; Appendix 2). Naturalness of sect. *Versicolores* is further supported by a particular pattern of seedling development (Champagnat, 1961).

Sects. *Diffusae* (*L. virgata* group), *Linaria* and *Speciosae*

Unlike sections *Macrocentrum*, *Pelisserianae*, *Versicolores* and *Lectoplectron* (= *Nuttallanthus*), the remaining four sections of *Linaria* were not resolved as monophyletic in our phylogenetic analyses. Relationships among species of sections *Linaria* (Figs. 1A-1C) and *Speciosae* (Figs. 1D-1F) were poorly resolved in clade E. Additionally, the BF tests yielded positive (for sect.

Linaria) and very strong (for sect. *Speciosae*) evidence against the monophyly of these sections (Table 5). Despite notable differences on seed shape (winged in sect. *Linaria* and wingless in sect. *Speciosae*), Sutton (1988) already indicated a close morphological relationship between species of both sections based on perennial habit, erect stems, and similar leaf, flower and capsule morphology. Four species of sect. *Diffusae* were also included in clade E (*L. virgata*, *L. albifrons*, *L. flava* and *L. triphylla*, henceforth "the *L. virgata* group"; Figs. 1G and 1H). The close relationship among the *L. virgata* group and sections *Linaria* and *Speciosae* is somewhat surprising. Nonetheless, the naturalness of section *Diffusae* has long been questioned. Valdés (1970) was the first author to suggest that this section was artificial and probably polyphyletic. Sutton (1988) also considered that this section was a heterogeneous group of species, including taxa that would have affected the delimitation of other sections. Moreover, this autor recognized the *L. virgata* group as a distinctive morphological complex within *Diffusae*. Unlike most other species of sect. *Diffusae*, this group displays very prominent anticlinal walls to the testa-cells and a sunken periclinal wall with no papilla. Since we retrieved low phylogenetic resolution within clade E and Bayes factors reflected positive, but not strong, evidence against the monophyly of section *Linaria* (of which only nine out of 45 species were sampled), additional markers and further taxon sampling are needed to reveal the phylogenetic relationships within this lineage.

Sects. *Diffusae* (*L. reflexa* group) and *Supinae*

Six of the remaining species of sect. *Diffusae*, including the type species *L. reflexa* (henceforth "the *L. reflexa* group"; Fig. 1I), formed a monophyletic group within clade F. This clade also included *L. latifolia* (sect. *Supinae*; Fig. 1K) and *L. hirta* (sect. *Diffusae*; Fig. 1J), which were resolved as basal to the other thirty-five species of sect. *Supinae*. *L. latifolia* is differentiated from the remaining species of sect. *Supinae* by several morphological features such as the erect stems, large flowers, broad leaves and long and slender calyx lobes. These peculiar traits of *L. latifolia* resemble those of sect. *Linaria* (stems and flowers) and sect. *Pelisserianae* (calyx lobes), although this species has been mostly confused with *L. hirta* because of their similar stems, leaves and flowers, being seed shape and inflorescence indumentum the main differences between both taxa.

Monophyly of the remaining species of sect. *Supinae* (excluding *L. latifolia*; Figs. 1L-1P) was obtained in all three phylogenetic analyses, although without strong statistical support. Naturalness of subsections *Supinae* and *Saxatile*, mainly differentiated by their wing width (Sutton, 1988), was clearly rejected in the consensus tree (Fig. 4) and BF tests (Table 5). Indeed, species relationships within clade F revealed the uselessness of wing shape (wing *vs.* marginal ridge or no wing) as a character dividing the section above the species level, as strong morphological differences in seed shape were found even in closely related species. No evident morphological synapomorphies were found for clade F. Although the presence of a fertile epicotylary stem together with heteromorphic –fertile and sterile– hypocotylary stems connects sections *Diffusae* and *Supinae* (Sutton, 1988), these traits are also found in *Diffusae* species of the unrelated *L. virgata* group (clade E).

Evolution of seed morphology

Viano's (1978b) hypothesis of *Linaria* evolution, i.e. a basal dichotomy in which species with wingless and winged seeds constitute two natural sister lineages, was clearly rejected by our results. Conversely, multiple shifts in seed morphology appear to have occurred in the course of *Linaria* evolution, as also indicated by the non-homologous anatomy of seed wings. Indeed, the seed wings which characterize sections *Pelisserianae*, *Linaria* and *Supinae* seem to have evolved independently, from wingless seeds at least in the last two groups, and most likely in the first one. Sutton (1988) pointed out that the seed wing of sect. *Pelisserianae* is not completely homologous to those of sections *Linaria* and *Supinae*, as a result of the exclusive symmetry pattern of sect. *Pelisserianae* seeds (see above). The same author hypothesized that, although seed wings of sections *Linaria* and *Supinae* are apparently homologous, they may not be uniquely derived, given the additional morphological characters that relate sect. *Linaria* to sect. *Speciosae*, and sect. *Supinae* to sect. *Diffusae*. This hypothesis is clearly supported by our results.

Finally, our analyses support recurrent shifts between broad wings and narrow wings (marginal ridges) or total lack of wings (*L. tursica*) in the course of the evolution of the *Supinae* lineage. Given the number of independent shifts (Fig. 6), this kind of residual wing, not clearly differentiated

from the seed disc, is shown as a poor character to support a systematic classification within this section.

Conclusions

Our ITS phylogeny provides the first phylogenetic insights into the evolution and infrageneric classification of *Linaria*. Monophyly of *Linaria* including Palearctic and American species is supported. The *Linaria* clade therefore constitutes a fourth New World-Old World lineage of Antirrhineae (see Vargas *et al.*, 2004). A basal divergence between species with winged and wingless seeds is clearly unsupported, given that seed wings appear to have evolved several times from wingless ancestors. Congruence between distinctive morphological characters and well-supported ITS lineages suggests that sections *Macrocentrum*, *Pelisserianae*, *Lectoplectron* and *Versicolores* constitute distinct evolutionary lineages and should be maintained in a phylogeny-based infrageneric classification of *Linaria*. On the other hand, our results cast doubt on the naturalness of sections *Supinae*, *Linaria*, *Speciosae* and *Diffusae*. Additional data will be required to support or reject the naturalness of sect. *Supinae*, and a proposal of merging sections *Linaria* and *Speciosae*. Polyphyly of sect. *Diffusae* had already been suggested based on morphology. Our phylogeny agrees with the recognition of at least two natural groups: the *L. reflexa* group, which would remain as sect. *Diffusae*; and the *L. virgata* group, which could be treated as sect. *Minutiflorae*, a taxon recognized by some authors and typified by *L. albifrons* (Bentham, 1846; Valdés, 1970). In any case, further molecular markers and analyses would be needed before firmly establishing a new sectional classification of all *Linaria* species.

Acknowledgements

We thank Emilio Cano for laboratory assistance; Beatriz Guzmán for assistance with ancestral state reconstructions; Llorenç Sáez and Benito Valdés for helpful comments on *Linaria* evolution and taxonomy; J.J. Aldasoro, S. and T. Taniguchi, B. Estébanez, J. Sánchez, the *Flora iberica* project, the MA, E, UPOS, SEV and ATH herbaria, and particularly the RNG herbarium and its curator S.L. Jury for plant material; the "Marismas del Odiel" natural reserve for collection permissions; J.

Ramírez and E. Sánchez-Gullón for field assistance; M. Luceño, O. Fragman-Sapir, N. V. Kurzenko, J. Ramírez, J. Quiles and G. D. Carr for permission to use their brilliant photographs; Y. Ruiz for assistance with scanning electron microscopy; and all previous authors who submitted Antirrhineae sequences to the GenBank database. This research was supported by the Spanish Ministry of Science and Innovation through project CGL2009-10031, by the Spanish Council of Scientific Research (CSIC) through a JAE-Pre fellowship to J.L. Blanco-Pastor, and by the Spanish Ministry of Education through a FPU fellowship (AP2007-01841) to M. Fernández-Mazuecos.

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Supporting Information

Table S1. Taxa, other than *Linaria* and *Nuttallanthus*, included in phylogenetic analyses of the Antirrhineae dataset, with geographic distributions, voucher specimens and GenBank accession numbers

Taxon	Distribution	Sampled locality	Voucher	GenBank accession no.
Acanthorrhinum Rothm. (1 sp.)	N Africa			
A. ramosissimum (Coss. & Durieu) Rothm.	N Africa	Morocco, road from Ouarzazate to Zagora	VAL 41469	AY731261
Albraunia Speta (3 spp.)	SW Asia			
A. foveopilosa Speta	SW Iran	Iran, Khuzistan, Baghmalek-Haftgel	TARI 38909	AY731250
Anarrhinum Desf. (8 spp.)	Europe, N Africa, and SW Asia			
A. corsicum Jordan & Fourr.	Corsica	France, Corsica	Podlech 47340 (A)	AF513881
A. bellidifolium (L.) Willd.	W Europe	Natural Botanical Garden of Dublin	VAL 145150	AY731263
Antirrhinum L. (25 spp.)	Western Mediterranean region			
A. australe Rothm.	S Spain	Spain, Granada, Castril	VAL 140895	AY731273
A. braun-blanquetii Rothm.	N Iberian Peninsula	Spain, Palencia, Cervera de Pisuerga	VAL 35121	AY731269
A. charidemi Lange	SE Spain	Spain, Almería, Cabo de Gata	VAL 37158	AY731282
A. cirrhigerum Welw. ex Ficalho	W Mediterranean region	Morocco, Doukkala-Abda, El Jadida	VAL 111299	EU677200
A. controversum Pau	SE Spain	Spain, Albacete, Villa de Ves	VAL 145152	AY731272
A. graniticum Rothm.	C Spain-E Portugal	Spain, Madrid, Fuentidueña del Tajo	VAL 99540	AY731283
A. grosii Font Quer	CW Spain	Spain, Ávila, Sierra de Gredos	VAL 37049	AY731281
A. hispanicum Chav.	S Spain	Spain, Granada, Veleta road	P. Vargas 120PV99	AY731286
A. latifolium Mill.	NE Spain to C Italy	Spain, Lérida, Bapà	VAL 144658	AY731274
A. linkianum Boiss.	Portugal	Portugal, Sintra	VAL 144655	AY731278
A. litigiosum Pau	SE Spain	Spain, Valencia, Serra	VAL 144656	AY731271
A. lopesianum Rothm.	NE Portugal	Portugal, Vimioso, Carçao	F. Amich & S. Bernardo s.n.	EU677217
A. majus L.	SW Europe	Spain, Lérida, Valle de Arán	VAL 144657	AY731280
A. meonanthum Hoffmanns. & Link	N Portugal	Spain, Ávila, El Tremedal	P. Vargas 149PV99	AY731284
A. microphyllum Rothm.	EC Spain	Spain, Guadalajara, Entrepeñas	VAL 40051	AY731267
A. molle L.	NE Spain	Spain, Huesca, Sopeira	VAL 35176	AY731268
A. mollissimum Rothm.	SE Spain	Spain, Almería, Sierra de Gádor	VAL 37143	AY731275
A. pertegasii Rothm.	E Spain	Spain, Castellón, Cova Fosca	J. Güemes JG4092	EU677226
A. pulverulentum Lázaro Ibiza	E Spain	Spain, Zaragoza, Nuévalos	VAL 31592	AY731279

Table S1. Continued.

A. sempervirens Lapeyr.	Pyrenees	Spain, Huesca, Panticosa	VAL 145148	AY731270
A. siculum Mill.	CE Mediterranean region	Italy, Sicily, Messina	VAL 119899	AY731276
A. subbaeticum Güemes, Mateu & Sánchez Gómez	SE Spain	Spain, Albacete, Bogarra, El Batán	J. Güemes JG4081	AY731287
A. tortuosum Bosc ex Vent.	CE Mediterranean region	Italy, Ancona, Sirolo	VAL 39871	AY731285
A. valentinum Font Quer	E Spain	Spain, Valencia, La Safor	VAL 39799	AY731266
Asarina Mill.	NE Spain-S France			
A. procumbens Mill.	NE Spain-S France	Botanischer Garten Berlin-Dahlem	VAL 145146	AF513879
Chaenorhinum (DC.) Rchb. (21 spp.)	Europe and Mediterranean region			
C. minus (L.) Lange	Europe-SW Asia	Unknown	McNeils 96-336 (GH)	AF513875
C. tenellum (Cav.) Lange	E Spain	Spain, Valencia, Moixent	VAL 37839	AY731251
Chelone L. (6 spp.)	N America			
C. obliqua L.	N America	Unknown	Wolfe 586 (OS)	AF375164
Cymbalaria Hill (9 spp.)	Europe, Mediterranean region, and SW Asia			
C. muralis G.Gaertn., B.Mey & Scherb.	S Europe	Switzerland	Nyffeler R. s.n.	AF513883
Epixiphium (A.Gray) Munz	SW North America			
E. wislizenii (A.Gray) Munz	SW North America	USA, Texas, El Paso Co.	UTEP 56828	AY878930
Galvezia Domb. ex Juss. (4 spp.)	S America			
G. ballii Munz	NW Perú	Perú, Piura	Cowan 4487 (TEX)	AY492104
G. fruticosa J.F.Gmel.	Perú	Unknown	Dillon 3776 (GH)	AF513885
Gambelia Nutt.	SW North America			
G. juncea (Benth.) D.A.Sutton	SW North America	Unknown	C.E. Freeman s.n.	AY316310
G. speciosa Nutt.	SW North America	Unknown	C.E. Freeman s.n.	AY316310
Globularia L. (22 spp.)	Europe, Mediterranean region, and SW Asia			
G. salicina Lam.	Canary Islands	Cultivated	Chase 2547 (K)	AF313039
Halleria L. (4 spp.)	Africa			
H. lucida L.	S Africa	Unknown	Wolfe 684 (OS)	AF375149
Holmgrenanthe Elisens	SW North America			
H. petrophila (Coville & C.V. Morton) Elisens	SW USA	USA, California, Inyo Co.	UTEP 67327	AY880231
Holzneria Speta (2 spp.)	SW Asia			
H. spicata (Korovin) Speta	SW Asia	Iran, Khorasan, Tobart-e Sefid	TARI 23577	AY731258
Howelliella Rothm. (1 spp.)	N America (California)			
H. ovata (Eastw.) Rothm.	SW North America	USA, California	Thompson 434 (GH)	AF513899
Isoplexis (Lindl.) Loudon (3 spp.)	Macaronesia			
I. canariensis (L.) Loud.	Canary Islands	Cultivated	Chase s.n. (K)	AF313033

Table S1. Continued.

Kickxia Dumort. (9 spp.)	Europe, N Africa, and W Asia			
K. elatine (L.) Dumort.	Eurasia and N Africa	Spain, Barcelona, Sant Pere de Ribes-Sitges	VAL 41793	AY731265
K. spuria (L.) Dumort.	Eurasia and N Africa	Spain, Valencia, Chera	VAL 37098	AY731264
Lafuentea Lag.	Western Mediterranean region			
L. rotundifolia Lag.	S Iberian Peninsula	Spain	Martínez Ortega 889 (SALA)	AF509816
Lophospermum D.Don (5 spp.)	N and C America			
L. erubescens D.Don	Mexico	Botanicher Garten Berlin-Dahlem	VAL 145154	AY731249
Mabrya Elisens	N Mexico, SW USA			
M. acerifolia (Pennell) Elisens	SW USA	USA, Arizona, Maricopa Co.	UTEP 56309	AY878934
Maurandella (A.Gray) Rothm. (1 spp.)	SW North America			
M. antirrhiniflora (Willd.) Rothm.	E Mexico	Mexico	Hill 18323 (GH)	AF513878
Maurandya Ortega	North and Central America			
M. scandens (Cav.) Pers.	C America	Cultivated	P. Vargas 1103	Forthcoming
Misopates Raf. (7 spp.)	Asia, Europe, and N Africa			
M. calycinum (Vent.) Rothm.	SW Iberian Peninsula and N Africa	Spain, Canary Islands, Lanzarote	ORT s/n	AY731259
M. orontium (L.) Raf.	Mediterranean region	Spain, Valencia, Serra	VAL 145155	AY731260
Mohavea A.Gray (2 spp.)	N America			
M. breviflora Coville	SW USA	Unknown	Hileman L. s.n.	AF513892
M. confertiflora A.Heller	SW North America	USA, California	Hileman L. s.n.	AF513891
Neogaerrhinum Rothm. (2 spp.)	SW North America			
N. filipes (A.Gray) Rothm.	SW North America	USA, California	Thompson 254 (GH)	AF513896
Plantago L. (270 spp.)	Cosmopolitan			
P. major L.	Eurasia	New Zealand	WELTU 20180	FJ024619
Pseudomisopates Güemes (1 spp.)	SW Europe			
P. rivas-martinezii (Sánchez Mata) Güemes	CW Spain	Spain, Ávila, Sierra de Gredos, Conventos creek	P. Vargas 377-99	AY731262
Pseudorontium (A.Gray) Rothm. (1 spp.)	SW North America			
P. cyathiferum (Benth.) Rothm.	SW North America	USA, California	Van Devender 92-268 (AZ)	AF513893
Rhodochiton Zucc. ex Otto & Dieta. (3 spp.)	C America			
R. atrosanguineum (Zucc.) Rothm.	C Mexico	Bergius Botanical Garden	VAL 145153	AF513876
Sairocarpus D.A.Sutton (13 spp.)	N America			
S. breweri (A.Gray) D.A.Sutton	SW USA	USA, California, Tehama Co.	UTEP 66786	AY880229
S. cornutus (Benth.) D.A.Sutton	SW USA	Unknown	Oyama RK 12 (A)	AF513905
S. costatus (Wiggins) D.A.Sutton	NW Mexico	Unknown	VanDevender 92-268 (AZ)	AF513893

Table S1. Continued.

S. coulterianus (A.DC.) D.A.Sutton	SW North America	Unknown	#16273 (RSA)	AF513890
S. kingii (S.Watson) D.A.Sutton	SW USA	Unknown	Morefield 3382 (GH)	AF513903
S. multiflorus (Pennell) D.A.Sutton	SW USA	Unknown	Oyama RK 5 (A)	AF513897
S. nuttalliamus D.A.Sutton	SW North America	USA, California	Oyama RK 27 (A)	AF513895
S. subcordatus (A.Gray) D.A.Sutton	SW USA	Unknown	Oyama RK 79 (A)	AF513902
S. vexillocalyculatus (Kellog) D.A.Sutton	SW USA	Unknown	Oyama RK 73 (A)	AF513900
S. virga (A.Gray) D.A.Sutton	SW USA	Unknown	Oyama RK 6 (A)	AF513898
S. watsonii (Vasey & Rose) D.A.Sutton	NW Mexico	Unknown	Fishbein 3136 (AZ)	AF513894
Schweinfurthia A.Braun (6 spp.)	Africa and SW Asia			
S. imbricata A.G.Mill., M.Short & D.A.Sutton	E Oman	Oman, Wadi Bed	E 99215	AY731254
S. latifolia Baker ex Oliver	Yemen	Yemen, Hadramout, Wadi 'Aidid	E 99214	AY731255
S. papilionacea (L.) Boiss.	SW Asia	Oman, Near Muscat	E 46435	AY731253
S. pedicellata (T. Anderson) Balf.f.	NE Africa-SW Asia	Socotra, Ras Bashorah	E 99213	AY731256
S. pterosperma (A. Rich.) A.Braun	NE Africa and SW Asia	Unknown	Thulin 8205 (UPS)	AF513882
S. spinosa A.G.Mill., M.Short & D.A.Sutton	Oman	Oman, Dhofar, Manston to Mudhai	E 99203	AY731257
Tetranema Benth. (10 spp.)	C America			
T. mexicanum Benth.	C America	Unknown	Wolfe s.n. (OS)	AF375151
Veronica L. (180 spp.)	Cosmopolitan			
Veronica officinalis	Eurasia	UK, Farthing Downs	Chase s.n. (K)	AF313012

Capítulo 3

Biogeografía de *Linaria* sect. *Versicolores*: aislamiento histórico frente a conexiones recientes a larga distancia entre Europa y África

Historical isolation *versus* recent long-distance connections between Europe and Africa in bifid toadflaxes (*Linaria* sect. *Versicolores*)

Una versión de este capítulo ha sido publicada en *PLoS ONE*:

Fernández-Mazuecos M, Vargas P (2011). Historical isolation *versus* recent long-distance connections between Europe and Africa in bifid toadflaxes (*Linaria* sect. *Versicolores*). *PLoS ONE* 6(7): e22234.

Abstract

Due to its complex, dynamic and well-known paleogeography, the Mediterranean region provides an ideal framework to study the colonization history of plant lineages. The genus *Linaria* has its diversity centre in the Mediterranean region, both in Europe and Africa. The last land connection between both continental plates occurred during the Messinian Salinity Crisis, in the late Miocene (5.96 to 5.33 Ma). Here we analyzed the colonization history of *Linaria* sect. *Versicolores* (bifid toadflaxes), which includes c. 22 species distributed across the Mediterranean, including Europe and Africa. Two cpDNA regions (*rpl*32-*trn*L^{UAG} and *trn*K-*mat*K) were sequenced from 66 samples of *Linaria*. We conducted phylogenetic, dating, biogeographic and phylogeographic analyses to reconstruct colonization patterns in space and time. Four major clades were found: two of them exclusively contained Iberian samples, while the other two included northern African samples together with some European samples. The bifid toadflaxes have been split in African and European clades since the late Miocene, and most lineage differentiation and speciation occurred during the Pliocene and Quaternary. We have strongly inferred four events of post-Messinian colonization following long-distance dispersal from northern Africa to the Iberian Peninsula, Sicily and Greece. The current distribution of *Linaria* sect. *Versicolores* lineages is explained by both ancient isolation between African and European populations and recent events of long-distance dispersal over sea barriers. This result provides new evidence for the biogeographic complexity of the Mediterranean region.

Introduction

Studying the role of biogeographic barriers as limiting factors for plant range expansion and gene flow allows investigation of the causes behind population differentiation and speciation (e.g. Garrick *et al.*, 2009; Jaramillo-Correa *et al.*, 2010). A remarkable spatial and temporal complexity makes the Mediterranean basin an ideal geographic framework for this approach. The abundance of islands, peninsulas, straits and mountains, and the complex history of climate and sea-level changes have created changing opportunities for plant dispersal and colonization across different barriers (Thompson, 1999; Comes, 2004). The Messinian Salinity Crisis (MSC), in the late Miocene (5.96 to 5.33 Ma BP; Krijgsman *et al.*, 1999) has long been considered the last major window of opportunity for plant colonization across the Mediterranean (Bocquet *et al.*, 1978). Desiccation of the Mediterranean Sea during this age formed land bridges that facilitated plant range expansion, including colonization events between Africa and Europe (e.g. Caujapé-Castells & Jansen, 2003). After the opening of the Strait of Gibraltar and the refilling of the Mediterranean basin (Miocene-Pliocene boundary, 5.33 Ma BP), isolation on both continental plates may have led to vicariant processes between European and African lineages. This intercontinental isolation made long-distance seed dispersal essential for range expansion over the newly created marine barriers.

Several factors may account for the differences in ability to expand a range over biogeographic barriers. Multiple patterns of colonization found in the Mediterranean suggest that habitat specificity, rather than morphological traits for dispersal, may have been crucial limiting factors (Piñeiro *et al.*, 2007; Rodríguez-Sánchez *et al.*, 2008; Guzmán & Vargas, 2009; Fernández-Mazuecos & Vargas, 2010). Certainly, recurrent seed colonization over sea barriers, specifically the Strait of Gibraltar, has been shown to be more likely if favourable ecological conditions are widespread, regardless of whether plants possess special mechanisms for long-distance dispersal (Guzmán & Vargas, 2009; Fernández-Mazuecos & Vargas, 2010). In other cases, however, long-term isolation between Iberian and NW African populations appears to have occurred, again irrespectively of seed dispersal mechanisms (Vargas *et al.*, 1999; Caujapé-Castells & Jansen, 2003; Terrab *et al.*, 2007; Cano-Maqueda *et al.*, 2008). Although the role of the Strait of Gibraltar as a biogeographic bridge or barrier has been assessed in several studies (see Rodríguez-Sánchez *et al.*, 2008 for a revision), little is still known about the impact of the

Mediterranean Sea as a large barrier for floristic exchange between Europe and Africa in the last 6 Ma (Lo Presti & Oberprieler, 2009; Yesson *et al.*, 2009; Fernández-Mazuecos & Vargas, 2010). Time-calibrated phylogenetic and phylogeographic analyses of Mediterranean plant lineages are required to understand dispersal, colonization and isolation processes across the changing sea barriers of the Mediterranean basin (Guzmán & Vargas, 2009; Fernández-Mazuecos & Vargas, 2010).

Toadflaxes (*Linaria* Mill.) constitute the largest genus within the tribe Antirrhineae. It comprises nearly 150 species classified into seven sections (see Table 1), and it has been suggested to be monophyletic by previous phylogenetic results (Vargas *et al.*, 2004). The genus has its diversity centre in the Mediterranean region, where all seven sections and c. 70% of species are present. Five sections are distributed both in the European and African parts of the Mediterranean region (Sutton, 1988). Small seeds of *Linaria* are enclosed in capsules, and may or may not be surrounded by an encircling wing. Therefore, this group constitutes a good system to analyze intercontinental colonization processes at the species and population levels, as well as the role of sea barriers in isolation.

The plastid genome (cpDNA) has been widely used in plant phylogenetics and phylogeography given its haploid and non-recombinant nature. When it is also maternally inherited, as commonly in angiosperms, including *Linaria* (Corriveau & Coleman, 1988), cpDNA lineages can be used to infer patterns of colonization by seeds (Schaal *et al.*, 1998). Recently developed methods, such as relaxed molecular-clock dating (Drummond *et al.*, 2006) and model-based biogeographic reconstruction (Ree & Smith, 2008; Ree & Sanmartín, 2009) allow estimating absolute dating of biogeographic events. Here, we applied a multi-scale approach based on the analysis of cpDNA sequences in order to reconstruct the colonization history of *Linaria* sect. *Versicolores* over marine barriers of the Mediterranean basin.

Materials and methods

Study taxa

Section *Versicolores* (Benth.) Wettst. represents one of the most distinctive subdivisions of *Linaria* (toadflaxes), due to the bifid style with discrete stigmatic areas (bifid toadflaxes), a trait not found in the rest of the genus (Viano, 1978a, b; Sutton, 1988). Seeds are wingless, and thus show no obvious capability for long-distance dispersal. Table 1 summarizes the infrasectional taxomony followed in this chapter, primarily based on Sutton (1988) (but see also Gómiz, 2004; Sáez & Bernal, 2009). The group comprises c. 22 species primarily of lowland habitats and is mainly distributed in the western Mediterranean, on both sides of the Mediterranean Sea (Europe/Africa). According to the most recent taxonomic revisions (Viano, 1978a, b; Sutton, 1988; Gómiz, 2004; Sáez & Bernal, 2009), sect. *Versicolores* includes eight European endemics, nine northern African endemics, one species from northern Africa and the Middle East (*L. tenuis*), and four species co-occurring in southern Europe and northern Africa: *L. incarnata*, *L. pedunculata* and *L. gharbensis* are found on both sides of the Strait of Gibraltar, while *L. multicaulis* is distributed in Morocco, Algeria, Tunisia, Sicily and Calabria. *Linaria hellenica*, an eastern Mediterranean species narrowly distributed in Greece, has been included within the African *L. tenuis* by some authors, on the basis of morphological characters (Tan & Iatrou, 2001). The taxonomic complexity of sect. *Versicolores* has long been recognized, particularly the poorly understood African taxa (Sutton, 1988). Therefore, species delimitation based on extant taxonomy must be taken with caution.

Sampling strategy and DNA sequencing

We sampled a total of 57 populations of *Linaria* sect. *Versicolores* (one individual per population), including representatives of 25 species and subspecies (Table 1). We failed to sample *L. dissita*. This is a poorly known African taxon of minor relevance for our objectives, because it seems to be closely related to other African species, with which it could even be con-specific (Sutton, 1988). We made special emphasis in the sampling of multiple populations of morphologically variable, widely distributed, and intercontinental species in order to test biogeographic

Table 1. Samples of the studied taxa and populations of *Linaria* sect. *Versicolores* and the outgroup including geographic distribution and *rpl*32-*trn*L^{UAG}/*trn*K-*mat*K sequence/haplotype codes (as in Figs. 2, 3, 4, 6).

Taxon	Distribution	Number of sampled populations	Sequence/ haplotype codes
Antirrhinum L.			
Antirrhinum graniticum Rothm.	-	1	-
Chaenorhinum (DC.) Rchb.			
Chaenorhinum macropodum (Boiss. & Reut.) Lange	-	1	-
Linaria Mill.			
Linaria sect. *Diffusae* (Benth.) Wettst.			
L. reflexa (L.) Chaz.	-	1	-
Linaria sect. *Linaria*			
L. vulgaris Mill.	-	1	-
Linaria sect. *Macrocentrum* D.A.Sutton			
L. chalepensis (L.) Mill.	-	1	-
Linaria sect. *Pelisserianae* Valdés			
L. triornithophora (L.) Willd.	-	1	-
Linaria sect. *Speciosae* (Benth.) Wettst.			
L. genistifolia (L.) Mill.	-	1	-
L. repens (L.) Mill.	-	1	-
Linaria sect. *Supinae* (Benth.) Wettst.			
L. alpina (L.) Mill.	-	1	-
L. amoi Campo ex Amo	-	1	-
L. micrantha (Cav.) Hoffmanns. & Link	-	1	-
Linaria sect. *Versicolores* (Benth.) Wettst.			
Subsect. *Versicolores*			
L. algarviana Chav.	SW Portugal (Algarve)	1	Ib6
L. bipartita (Vent.) Willd.	W Morocco	2	17 (x2)
L. bordiana Santa & Simonneau	NW Africa	2	13, 14
L. clementei Haensel. ex Boiss.	S Spain (Málaga)	2	Ib2, Ib1
L. gharbensis Batt. & Pit.	NW Africa, SW Spain	4	5, 6, 7, 16
L. hellenica Turrill	S Greece	1	4
L. imzica Gómiz	S Morocco (Anti Atlas)	1	21
L. incarnata (Vent.) Spreng.	SW Iberian Peninsula, NW Morocco	6	Ib6 (x2), Ib7, 7, 8, 23
L. maroccana Hook.f.	Morocco (mainly High Atlas)	2	22, 23
L. multicaulis (L.) Mill.			
subsp. *multicaulis*	Sicily, S Italy (Calabria)	1	12
subsp. *aurasiaca* (Pomel) D.A.Sutton	Tunisia, NE Algeria	1	11
subsp. *galioides* (Ball) D.A.Sutton	Morocco (High Atlas)	2	2, 3
subsp. *heterophylla* (Desf.) D.A.Sutton	NW Africa	5	1, 16, 18, 19, 20
L. pedunculata (L.) Chaz.	S Iberian Peninsula, NW Africa, Balearic islands	9	9 (x9)
L. pinifolia (Poir.) Thell.	Tunisia, Algeria	1	16
L. pseudoviscosa Murb.	Tunisia	1	10
L. salzmannii Boiss.	S Spain (Málaga)	1	Ib1
L. spartea (L.) Chaz.	Iberian Peninsula, S France	2	Ib3, Ib6
L. tenuis (Viv.) Spreng.	N Africa, Middle East	2	11 (x2)
L. tingitana Boiss. & Reut.	NW Africa	2	15, 16
L. viscosa (L.) Chaz.			
subsp. *viscosa*	S Iberian Peninsula	2	Ib4, Ib5
subsp. *spicata* (Coutinho) D.A.Sutton	SE Iberian Peninsula	2	Ib1 (x2)
L. weilleri Emb. & Maire	S Morocco (Anti Atlas)	1	21
Subsect. Elegantes (Viano) D.A.Sutton			
L. elegans Cav.	NW Iberian Peninsula	2	Le1, Le2
L. nigricans Lange	SE Spain (Almería)	2	Ln1, Ln2

hypotheses. To test the monophyly of section *Versicolores*, we also sampled nine additional species representing the remaining six sections of *Linaria*. One species of *Chaenorhinum* and one of *Antirrhinum* were included as the outgroup on the basis of a previous phylogeny of the tribe Antirrhineae (Vargas *et al.*, 2004). Plant material was collected in the field and dried in silica gel or obtained from herbarium collections (RNG, MA, ATH, UPOS, SALA; Supporting Information Table S1).

Total genomic DNA was extracted using DNeasy Plant Mini Kit (QIAGEN Inc., California). A pilot study using 6 samples of different species was performed to find the most variable sequences among 14 plastid DNA regions previously used in phylogenetic and phylogeographic analyses (Shaw *et al.*, 2005; Shaw *et al.*, 2007). DNA regions were amplified in an Eppendorf Mastercycler Epgradient S (Westbury, NY) or a MJ Research PTC-200 (Massachusetts) thermal cycler. After 1 min pretreatment at 95ºC, PCR conditions were: 30 cycles of 1 min at 94ºC, 1–2 min at 48–55ºC and 1–2 min at 72ºC. In certain reactions, a volume of 1 μL of bovine serum albumine (BSA) at 1 mg ml^{-1} was included in each 25 mL reaction to improve the efficiency of amplification. Amplified products were treated with ExoSAP-IT (USB Corporation, Ohio) and submitted to Macrogen Inc. (Seoul, South Korea) for sequencing. Resulting sequence data were assembled and edited using Geneious Pro v5 (Drummond *et al.*, 2010). We identified two highly variable cpDNA regions: *rpl*32-*trn*L^{UAG} (Shaw *et al.*, 2007) and *trn*K-*mat*K (Johnson & Soltis, 1994) and then the sequencing of these regions was extended to every sampled individual. In order to facilitate amplification and sequencing from partially degraded DNA obtained from herbarium specimens, we designed the following internal primers for both regions and used them in combination with the standard primers: rpl32-trnL_intF (5' - CATTTCCAAGGTGGGGAGTCT - 3'), rpl32-trnL_intR (5' - AGAAATAGGTTGATGGGGA - 3'), trnK-matK_intF1 (5' - ACCTGTCTCCGAGGTATCTA - 3'), trnK-matK_intF2 (5' - GGGGTTTGCATTTATTGTGG - 3'), trnK-matK_intR1 (5' - CACGATCATGAGCAAACGCA - 3'), and trnK-matK_intR2 (5' - CCACAATAAATGCAAACCCC - 3'). We also designed a reverse primer specific to *Linaria* sect. *Versicolores* for *trn*K-*mat*K: 1470R_Lvers (5'-AAGATGTTGATCGTAAATCC-3'). All sequences were submitted to GenBank (see Supporting Information Table S1 for accession numbers).

Phylogenetic analysis

Sequences of each cpDNA region (*rpl*32-*trn*L^{UAG} and *trn*K-*mat*K) were aligned using MAFFT 6 (Katoh *et al.*, 2002) with default parameters, and further adjustments were made by visual inspection. The two regions were combined in a single matrix, and phylogenetic relationships were assessed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). The MP analysis was performed in TNT 1.1 (Goloboff *et al.*, 2003) using a heuristic search with 10,000 replicates saving two most-parsimonious trees per replicate, followed by a second heuristic search retaining all best trees and using the trees obtained in the previous 10,000 replicates as the starting ones. Bootstrap support (MP-BS) of clades was assessed using 1000 standard replicates. For ML and BI analyses, the simplest model of sequence evolution that best fits the sequence data (GTR for *trn*K-*mat*K and GTR+G for *rpl*32-*trn*L^{UAG} and the combined dataset) was determined under the Akaike Information Criterion (AIC) in jModelTest 0.1.1 (Posada, 2008). ML was implemented in PhyML 3.0 (Guindon & Gascuel, 2003) with 500 non-parametric bootstrap replicates (ML-BS). BI was performed in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) using two searches with 10 million generations each and a sample frequency of 1000. The two regions were partitioned and unlinked. Chain convergence was assessed with Tracer 1.4 (Rambaut & Drummond, 2007), and a 50% majority rule consensus tree with Bayesian posterior probabilities (PP) of clades was calculated to obtain the Bayesian estimate of phylogeny after removing the first 10% generations as burn-in.

Estimation of divergence times

To estimate divergence times among *Linaria* sect. *Versicolores* lineages, we implemented a relaxed molecular-clock approach in BEAST v.1.6.0 (Drummond *et al.*, 2006; Drummond & Rambaut, 2007), a software that simultaneously estimates tree topology and node ages. Identical sequences of the *rpl*32-*trn*L^{UAG}/*trn*K-*mat*K matrix were removed from the analysis. Gaps were treated as missing data. Since no reliable fossils of *Linaria* are known to date, only molecular estimates were available for temporal calibration of the tree. The divergence time between *Chaenorhinum* and *Linaria* was modelled as a normal distribution with mean = 29 Ma and standard deviation = 4.6, on the basis of an estimate obtained in a relaxed molecular-

clock analysis of tribe Antirrhineae (P. Vargas *et al.*, unpublished). This analysis incorporates a calibration of 97 Ma for the crown-age of Lamiales (Bremer *et al.*, 2004) and minimum stem-age constraints for Lamiales families and tribes based on five fossils: *Fraxinus wilcoxiana* (Oleaceae, Middle Eocene) (Call & Dilcher, 1992), *Catalpa rugosa* (Bignoniaceae, Early-Middle Oligocene) (Reid & Chandler, 1926), *Ajuginucula smithii* (Lamiaceae, Early-Middle Oligocene) (Reid & Chandler, 1926), *Gratiola tertiaria* (Gratioleae, Miocene) (Łańcucka-Środoniowa, 1977) and *Plantaginacearumpollis* (Plantaginaceae *s.s.*, Middle Miocene) (Nagy, 1963). All these fossils have been considered reliable and proposed as calibration points for molecular dating in previous studies (Besnard *et al.*, 2009; Martínez-Millán, 2010; Thiv *et al.*, 2010). The substitution rate variation was modelled using an uncorrelated lognormal distribution, and a birth-death process (Gernhard, 2008) was employed as tree prior. Two MCMC analyses were run for 10 million generations, with a sample frequency of 1000. Both chains were combined using LogCombiner 1.4.8, after discarding the first 10% of sampled generations as burn-in. Parameter analysis in Tracer 1.4 (Rambaut & Drummond, 2007) confirmed adequate sample size, with ESS values above 650 and plots showing equilibrium. Trees were summarized in a maximum clade credibility (MCC) tree obtained in TreeAnotator 1.4.8 and visualized in FigTree 1.1.2.

Biogeographic reconstruction

In order to infer colonization events of *Linaria* sect. *Versicolores* across the Mediterranean, biogeographic reconstructions were conducted delimiting four areas based on the distribution of sampled taxa and the presence of marine barriers: northern Africa (A); Iberian Peninsula (I); Sicily (S); and Greece (G). We employed a model-based maximum-likelihood approach for ancestral area optimization: the dispersal-extinction-cladogenesis (DEC) model implemented in Lagrange 2.0.1 (Ree & Smith, 2008). This analysis requires a fully dichotomous tree, and thus the BEAST output is appropriate. Given the polyphyly of several species in the phylogenetic analysis of the full dataset (see below), we did not attempt a biogeographic reconstruction using the complete phylogeny and species distribution ranges. Instead, we employed the phylogeny (MCC tree) obtained in the BEAST analysis. Outgroup taxa were pruned, and distribution ranges of plants containing the same sequence, instead of species ranges, were

attached to tree tips. Although the inclusion of an outgroup has been recommended for DEC analysis (Kodandaramaiah, 2010), we did not proceed because poor sample of *Linaria* as a whole impeded finding a reliable sister group to sect. *Versicolores*.

Lagrange uses DEC modelling to compute the likelihood of range inheritance scenarios at nodes in a phylogeny, and allows incorporation of information about changing dispersal opportunities associated to geological events (e.g. area connections). We compared four models (M0-M3) differing in maximum number of areas allowed in ancestral ranges and constancy of dispersal rates through time. In M0, the maximum number of areas was unconstrained and dispersal rate was constant through time. M1 incorporated a maximum of two areas in ancestral ranges, based on current distributions. In M2, dispersal rate was set to vary according to historical connections among areas: it was maximum ($\lambda_D = 1$) during the MSC (5.96-5.33 Ma), when the contact between the Eurasian and African plates and the desiccation of the Mediterranean Sea eliminated marine barriers among areas; conversely, dispersal rate was set to a lower value ($\lambda_D = 0.1$) during the time intervals before and after the Messinian event, when marine barriers were active. Finally, model M3 combined constraints on maximum number of areas and dispersal rates from M1 and M2. To assess the statistical significance of likelihood differences among models, we employed the conventional cut-off value of two log-likelihood units (Ree *et al.*, 2005).

Reconstructions described above rely on the single MCC tree. To account for uncertainty in tree topology and node ages, possibly affecting the reconstruction at the root node, we repeated the analyses under the four DEC models over a sample of 100 trees from the posterior distribution of the BEAST analysis. Then we summarized the resulting scenarios of range inheritance at the root node obtained in the 100 analyses under each DEC model.

For comparison with a parsimony-based reconstruction method, we also performed dispersal-vicariance analyses (DIVA) (Ronquist, 1997). To account for phylogenetic uncertainty and uncertainty in area optimization, DIVA analyses were implemented in S-DIVA (Yu *et al.*, 2010), a software that statistically evaluates the alternative ancestral ranges at nodes based on a set of trees (Nylander *et al.*, 2008; Harris & Xiang, 2009). As input, we used the complete tree distribution obtained from BEAST and the final MCC tree. We conducted two analyses allowing for a maximum of two or four areas in ancestral ranges and with the option "Allow reconstruction"

in effect, which calculates the probabilities of ancestral ranges at nodes following Harris & Xiang (2009). The two analyses were repeated unchecking the mentioned option, thus applying the method of Nylander *et al.* (2008).

Haplotype data analysis

We analyzed the colonization history of intercontinental lineages through a haplotype network approach. Genealogical relationships among haplotypes of clades III and IV (see below) were inferred using the statistical parsimony algorithm (Templeton *et al.*, 1992), as implemented in TCS 1.21 (Clement *et al.*, 2000). The maximum number of differences resulting from single substitutions among haplotypes was calculated with 95% confidence limits, treating gaps as missing data.

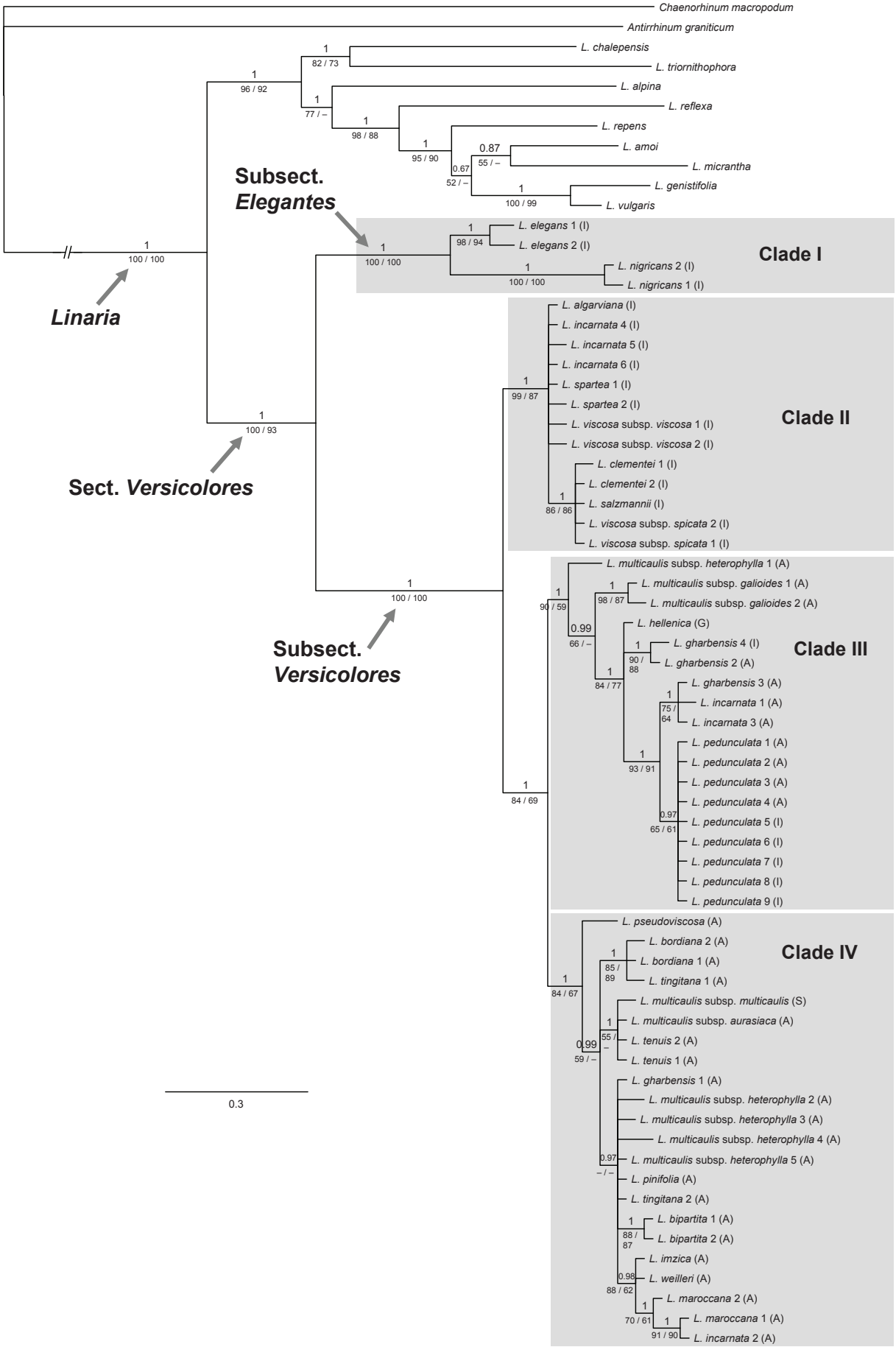
Results

Phylogenetic relationships

Two of the 14 variable cpDNA regions tested (*rpl*32-*trn*L^{UAG} and *trn*K-*mat*K) rendered the highest number of reliable nucleotide substitutions. The characteristics of the two sequenced cpDNA regions are summarized in Table 2. The total aligned length of the combined dataset was 2066 bp, and 187 of the 395 variable sites were parsimony-informative. The 50% majority-rule consensus tree of the Bayesian analysis is shown in Fig. 1. The ML tree showed the same

Table 2. Characteristics of the *rpl*32-*trn*L^{UAG} and *trn*K-*mat*K sequences obtained for *Linaria* sect. *Versicolores* samples and the outgroup.

	***rpl*32-*trn*L^{UAG}**	***trn*K-*mat*K**
Aligned length (bp)	830	1236
Ungapped length range	568-754	1209-1227
Pairwise % identity	94.7	98.3
Variable characters	209	186
Parsimony-informative characters	105	82
Mean % G+C content	22.2	32.3


Chaenorhinum macropodum
Antirrhinum graniticum
L. chalepensis
L. triornithophora
L. alpina
L. reflexa
L. repens
L. amoi
L. micrantha
L. genistifolia
L. vulgaris
Subsect. Elegantes
L. elegans 1 (I)
L. elegans 2 (I)
L. nigricans 2 (I)
L. nigricans 1 (I)
Clade I
Linaria
Sect. Versicolores
L. algarviana (I)
L. incarnata 4 (I)
L. incarnata 5 (I)
L. incarnata 6 (I)
L. spartea 1 (I)
L. spartea 2 (I)
L. viscosa subsp. viscosa 1 (I)
L. viscosa subsp. viscosa 2 (I)
L. clementei 1 (I)
L. clementei 2 (I)
L. salzmannii (I)
L. viscosa subsp. spicata 2 (I)
L. viscosa subsp. spicata 1 (I)
Clade II
Subsect. Versicolores
L. multicaulis subsp. heterophylla 1 (A)
L. multicaulis subsp. galioides 1 (A)
L. multicaulis subsp. galioides 2 (A)
L. hellenica (G)
L. gharbensis 4 (I)
L. gharbensis 2 (A)
L. gharbensis 3 (A)
L. incarnata 1 (A)
L. incarnata 3 (A)
L. pedunculata 1 (A)
L. pedunculata 2 (A)
L. pedunculata 3 (A)
L. pedunculata 4 (A)
L. pedunculata 5 (I)
L. pedunculata 6 (I)
L. pedunculata 7 (I)
L. pedunculata 8 (I)
L. pedunculata 9 (I)
Clade III
L. pseudoviscosa (A)
L. bordiana 2 (A)
L. bordiana 1 (A)
L. tingitana 1 (A)
L. multicaulis subsp. multicaulis (S)
L. multicaulis subsp. aurasiaca (A)
L. tenuis 2 (A)
L. tenuis 1 (A)
L. gharbensis 1 (A)
L. multicaulis subsp. heterophylla 2 (A)
L. multicaulis subsp. heterophylla 3 (A)
L. multicaulis subsp. heterophylla 4 (A)
L. multicaulis subsp. heterophylla 5 (A)
L. pinifolia (A)
L. tingitana 2 (A)
L. bipartita 1 (A)
L. bipartita 2 (A)
L. imzica (A)
L. weilleri (A)
L. maroccana 2 (A)
L. maroccana 1 (A)
L. incarnata 2 (A)
Clade IV
0.3

topology, while the strict consensus tree of the MP analysis was fully congruent, although with a lower resolution and support values (Fig. 1).

All three phylogenetic analyses recognized section *Versicolores* as monophyletic with high support values. Within the section, two well-supported sister clades were retrieved, which supported the two morphology-based subsections: *Elegantes*, formed by the two sister species *L. elegans* and *L. nigricans* (clade I; PP = 1; ML-BS = 100%; MP-BS = 100%); and *Versicolores*, encompassing the remaining species (PP = 1; ML-BS = 100%; MP-BS = 100%). Two major lineages were found within the latter subsection. The first one (clade II; PP = 1; ML-BS = 99%; MP-BS = 87%) contained Iberian samples, including all the Iberian endemics, *L. spartea* and Iberian accessions of *L. incarnata*, and was sister to a second lineage formed by clades III and IV (PP = 1; ML-BS = 84%; MP-BS = 69%). These two clades primarily contained northern African samples (including those of *L. incarnata*), together with samples from Sicily and Greece, and Iberian samples of *L. gharbensis* and *L. pedunculata*. Accessions of the same species or subspecies were retrieved as monophyletic groups only for *L. elegans*, *L. nigricans*, *L. multicaulis* subsp. *galioides*, *L. pedunculata* and *L. bipartita*, while polyphyly was clearly retrieved for *L. incarnata*, *L. gharbensis*, *L. multicaulis* subsp. *heterophylla* and *L. tingitana*.

Divergence times

Values of standard deviation of the uncorrelated lognormal relaxed clock (0.25) and coefficient of variation (0.24) for rate heterogeneity within our cpDNA dataset indicated a low deviation from a strict molecular clock. The topology of the MCC tree (Fig. 2) was congruent with that of the other phylogenetic analyses. The chronogram supported a crown-age for *Linaria* sect.

Fig. 1. Phylogenetic relationships of 57 samples representing 25 species and subspecies of *Linaria* sect. *Versicolores* based on the combined analysis of cpDNA regions *rpl*32-*trn*L^{UAG} and *trn*K-*mat*K. The majority-rule consensus tree obtained in the Bayesian analysis is shown. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood / maximum parsimony bootstrap values. A hyphen (-) indicates no bootstrap support over 50%. Populations of the same species are numbered as in Table S1. Geographic location of sect. Versicolores samples is shown in brackets. I – Iberian Peninsula; A – northern Africa; S – Sicily; G – Greece.

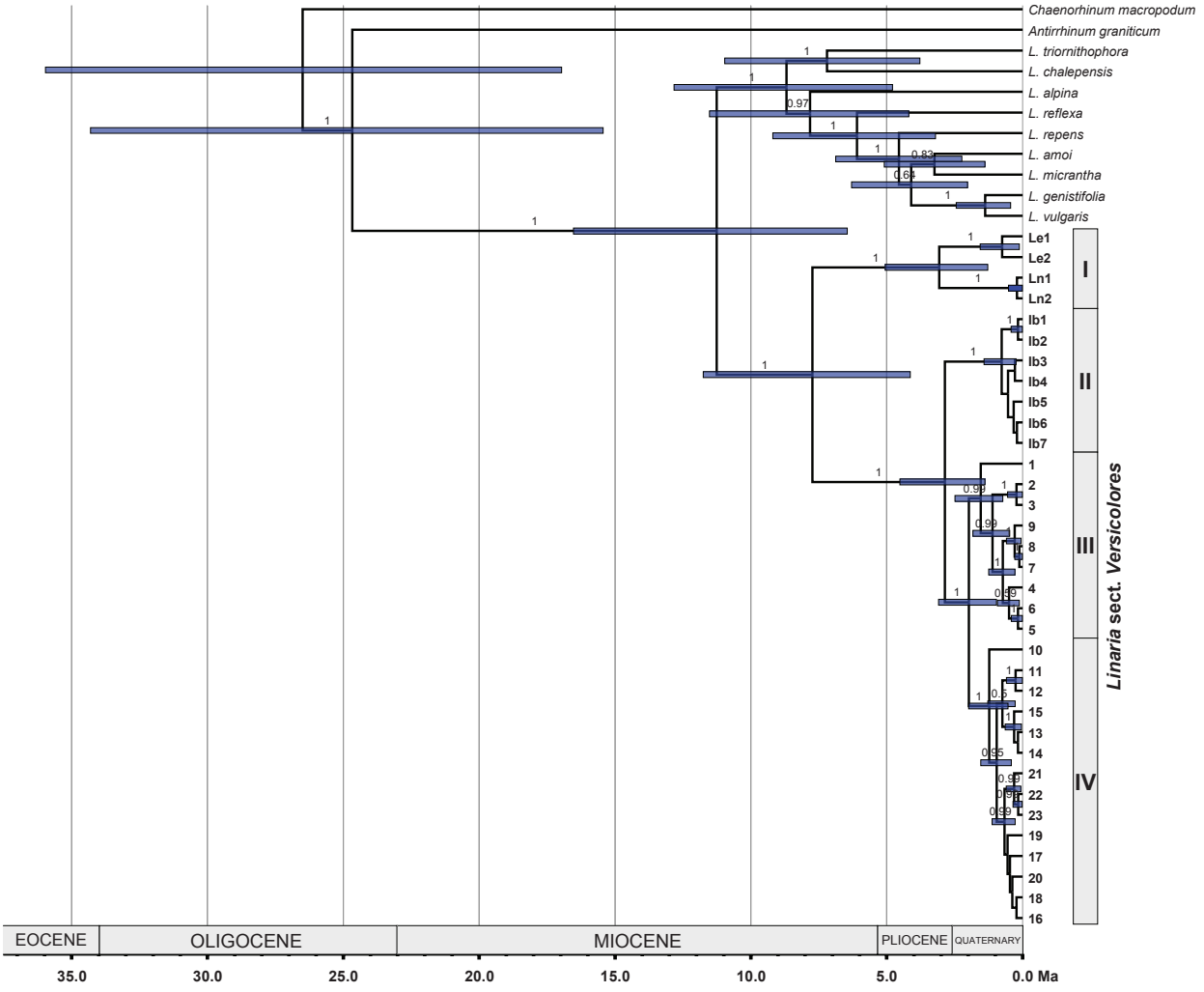


Fig. 2. Maximum clade credibility tree produced by relaxed molecular-clock analysis of *rpl*32-*trn*L^{UAG} and *trn*K-*mat*K sequences in BEAST, excluding sequence identities of *Linaria* sect. *Versicolores* and the outgroup. Node bars represent the 95% highest posterior density intervals for the divergence time estimates of clades with posterior probabilities above 0.50. Values above branches indicate Bayesian posterior probabilities. Major clades of the study group are indicated.

Table 3. Divergence dates of major plastid clades of *Linaria* sect. *Versicolores*, presented as mean/median crown ages plus 95% highest posterior density (HPD) intervals based on relaxed molecular-clock analysis of *rpl*32-*trn*L^{UAG} and *trn*K-*mat*K sequences in BEAST.

Clade	Crown age (Ma BP)	95% HPD interval
Sect. *Versicolores*	7.73 / 7.60	4.13 – 11.75
Subsect. *Elegantes* (clade I)	3.06 / 2.94	1.28 – 5.05
Subsect. *Versicolores* (clade II+III+IV)	2.86 / 2.77	1.39 – 4.51
Clade II	0.77 / 0.71	0.24 – 1.42
Clade III+IV	1.97 / 1.91	0.96 – 3.09
Clade III	1.54 / 1.49	0.72 – 2.49
Clade IV	1.23 / 1.19	0.54 – 1.98

Versicolores around the late Miocene, while a Pliocene or early Quaternary divergence for the two main lineages of subsect. *Versicolores* (Fig. 2; Table 3). Lineage differentiation within clades II, III and IV appears to have occurred during the Quaternary. A very recent divergence (< 1 Ma) was supported for European accessions (haplotypes 4, 6, 9 and 12) within the mainly northern African clades (III, IV).

Biogeographic reconstruction

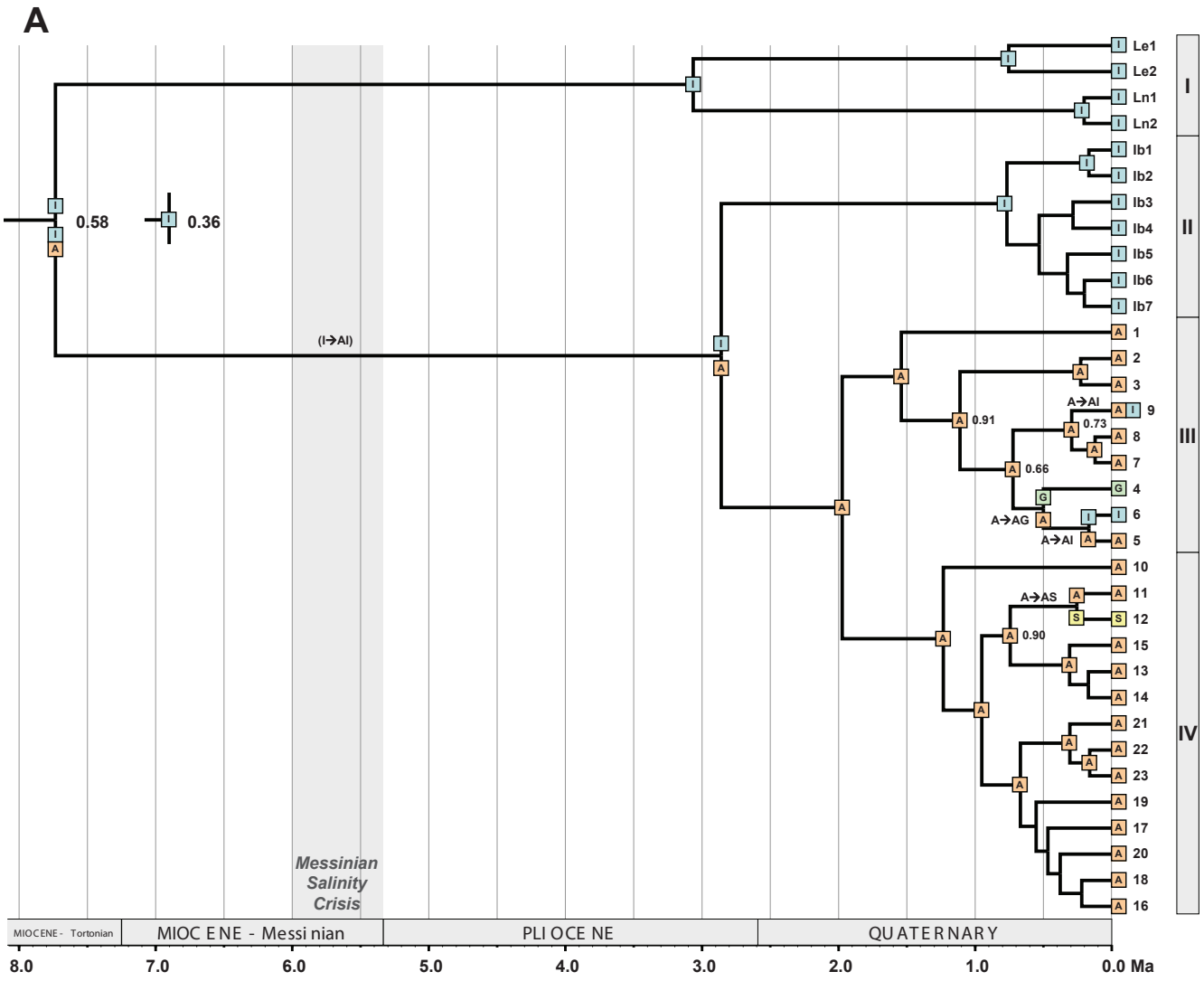
The four biogeographic models tested in Lagrange (Table 4) produced results with similar likelihood values. Model M2 received the highest log-likelihood (-28.28), but all other models fell within 2 log-likelihood units of the optimal one, and thus M2 was not significantly supported as the best model. The four models produced slightly different reconstructions of colonization history, with models M0 and M2 (both with unconstrained maximum number of areas in

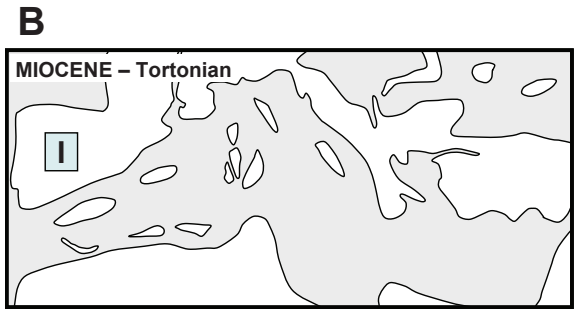
Table 4. Results for the biogeographic models tested in Lagrange, including values of log-likelihood (lnL), dispersal rate (λ_D), extinction rate (λ_E) and maximum-likelihood scenarios of range inheritance for the tree root and selected clades. When a bar separates two ranges, the first range is inherited by the upper daughter branch in Fig. 3 and the second range is inherited by the lower daughter brach. If a node has multiple scenarios within 2 log-likelihood units of the optimal reconstruction, the two most likely scenarios are shown, and the relative probability of each is indicated in brackets. I – Iberian Peninsula; A – northern Africa.

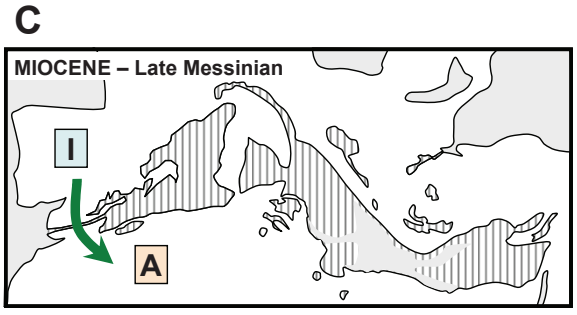
	M0	**M1**	**M2**	**M3**
lnL	-28.57	-28.75	-28.28	-28.65
λ_D	0.038	0.046	0.425	0.493
λ_E	0.000	0.000	0.000	0.000
Sect. *Versicolores* (root)	I (0.50), I|AI (0.37)	I|AI (0.46), I (0.46)	I|AI (0.51), I (0.39)	I|AI (0.58), I (0.36)
Subsect. *Elegantes* (clade I)	I	I	I	I
Subsect. *Versicolores* (clade II+III+IV)	I|A (0.80), I|AI (0.07)	I|A	I|A (0.82), I|AI (0.06)	I|A
Clade II	I	I	I	I
Clade III+IV	A (0.79), AI|A (0.10)	A	A (0.81), AI|A (0.10)	A
Clade III	A (0.79), A|AI (0.09)	A	A (0.81), A|AI (0.10)	A
Clade IV	A	A	A	A

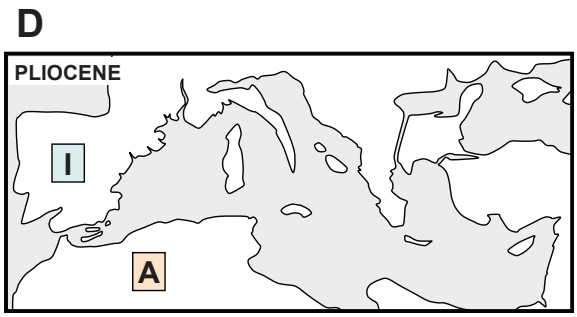
ancestral ranges) resulting in a higher number of alternative scenarios falling within 2 log-likelihood units of the optimal reconstruction. For example, seven alternative scenarios were obtained for the most recent common ancestor of sequences 4-9 in models M0 and M2, while three and four scenarios were obtained for models M1 and M3 respectively, even though all four models inferred an African ancestor (A|A, being the area on the left the one inherited by the upper daughter branch in Fig. 3, and the area on the right the one inherited by the lower daughter branch) as the optimal reconstruction. For simplicity, we show the optimal reconstruction under the most biologically realistic model (M3, higher dispersal rate during the MSC and a maximum of two ancestral areas at nodes) in Fig. 3A. In fact, the four models inferred the same optimal reconstruction in all but two nodes: the root node, as discussed below, and the most recent common ancestor of sequences 4-6. In the latter, G|AI was the optimal scenario in models M0 and M2, while G|A was for models M1 and M3, thus placing a dispersal event to the Iberian Peninsula along different branches.

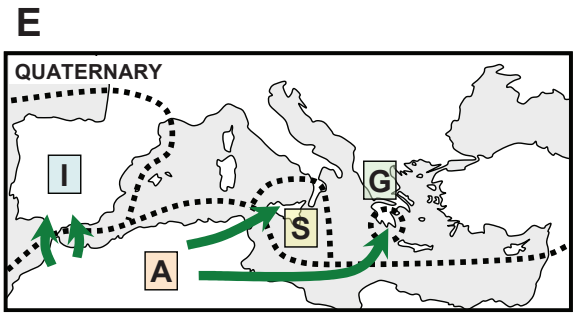
Fig. 3. Hypothesis of colonization history based on DEC analysis. (A) Biogeographic reconstruction based on dispersal-extinction-cladogenesis modelling implemented in Lagrange using the single MCC tree from the BEAST analysis (Fig. 2) after pruning outgroup taxa. Coloured squares represent maximum-likelihood range inheritance scenarios reconstructed under model M3 for nodes with PP above 0.5. Ranges inherited from widespread ancestors following cladogenesis are shown at the base of diverging branches, while single-area ancestral ranges are shown at nodes. Inferred events of dispersal along branches are also illustrated. When a node has alternative scenarios within 2 log-likelihood units of the optimal reconstruction, the relative probability (fraction of the global likelihood) for the optimal reconstruction is indicated. Given the relevance of the root node for the early colonization history of the group, the two alternative reconstructions are displayed, and the dispersal event inferred under the second best scenario (relative probability 0.36) is shown in brackets along the branch leading to subsect. Versicolores. (B-D) Hypothesis of colonization history of *Linaria* sect. *Versicolores* across the Mediterranean basin since the Late Miocene, based on phylogenetic, dating and biogeographic reconstruction results, as well as geological information. Coloured squares represent the range occupied by the group during each period, and arrows indicate hypothetical colonization events. Paleogeographic maps are based upon Jolivet *et al.* (2006) (white – emerged areas; grey – submerged areas; lined – desiccating areas). Areas delimited for reconstructions are displayed in E. I – Iberian Peninsula; A – northern Africa; S – Sicily; G – Greece.


A
Le1
Le2
Ln1
Ln2
Ib1
Ib2
Ib3
Ib4
Ib5
Ib6
Ib7
I
II
III
IV
0.58
0.36
(I→AI)
0.91
0.73
0.66
0.90
A→AI
A→AG
A→AS
Messinian Salinity Crisis
MIOCENE - Tortonian
MIOCENE - Messinian
PLIOCENE
QUATERNARY
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0 Ma


B
MIOCENE – Tortonian
I


C
MIOCENE – Late Messinian
I
A


D
PLIOCENE
I
A


E
QUATERNARY
I
G
S
A

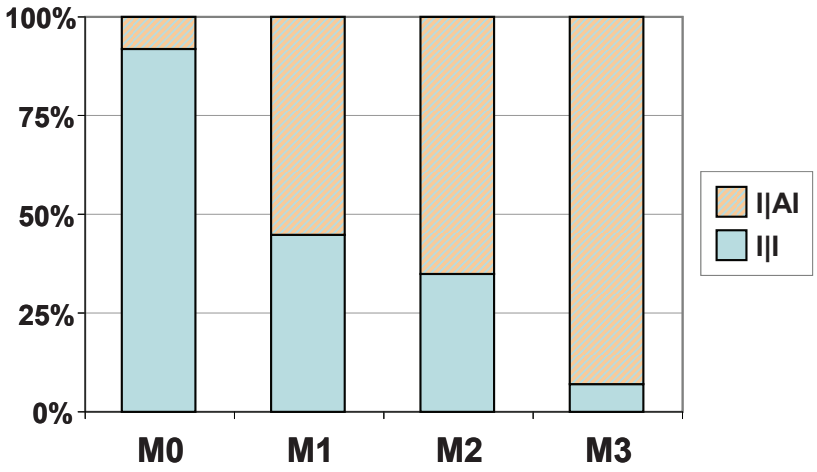


Fig. 4. Uncertainty of area reconstruction at the root. A sample of 100 trees from the posterior distribution of the BEAST analysis was analyzed in Lagrange under the four DEC models. Bars summarize the proportion of trees yielding a certain maximum-likelihood scenario for the root node. Only two possible scenarios were retrieved: I|AI and I|I.

The optimal reconstruction under model M3 (Fig. 3A) supported a common ancestor of sect. *Versicolores* distributed both in the Iberian Peninsula and northern Africa (relative probability 0.58). An Iberian-only range was inherited by one of its daughter lineages, leading to the common ancestor of subsect. *Elegantes* (clade I), while a widespread range (IA) was inherited by the daughter lineage leading to subsect. *Versicolores* (clades II-IV). In the cladogenetic event at the base of subsect. *Versicolores*, this widespread ancestor yielded two daughter lineages inheriting mutually exclusive ranges: Iberian Peninsula for the ancestor of clade II and northern Africa for the ancestor of clades III-IV. Subsequent dispersal events from northern Africa to the Iberian Peninsula and Greece in clade III, and to Sicily in clade IV gave rise to current ranges of sublineages and species in these lineages.

Under the second best scenario at the root node (relative probability 0.36), an Iberian common ancestor of sect. *Versicolores* produced two Iberian daughter lineages, one of which dispersed to northern Africa, giving rise to a widespread western Mediterranean ancestor of subsect. *Versicolores* (Fig. 3A). The uncertainty on the range of the common ancestor of sect. *Versicolores* was maintained under the other DEC models, with an Iberian ancestor supported by model M0 and a widespread ancestor by models M1 and M2 (Table 4). When taking into account the uncertainty on topology and branch lengths by analyzing 100 trees from the posterior distribution of the BEAST analysis (Fig. 4), we obtained contrasting results under different DEC

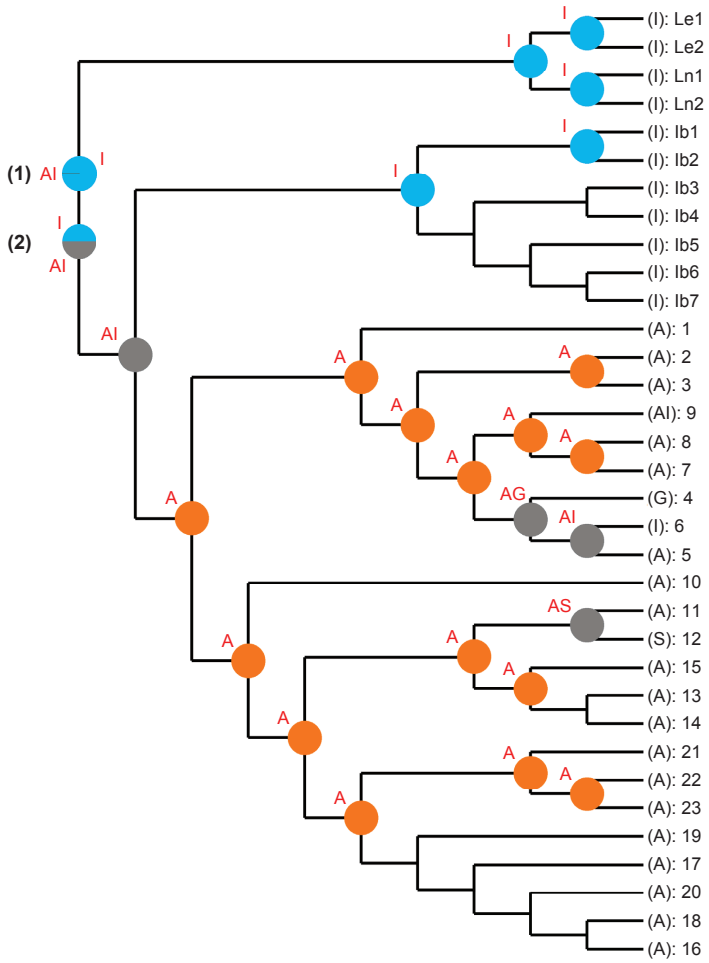


Fig. 5. Biogeographic reconstruction based on statistical dispersal-vicariance analysis as implemented in S-DIVA, with the maximum number or areas at ancestral nodes set to two. The tree is the same as in Fig. 2 after pruning outgroup taxa. Pie charts at nodes represent marginal probabilities for ancestral areas. Different ways of calculating probabilities of ancestral ranges did not affect the result, except for the root node, where reconstructions following Harris & Xiang (2009) (1) and Nylander *et al.* (2008) (2) are shown. I – Iberian Peninsula; A – northern Africa; S – Sicily; G – Greece.

models. An Iberian ancestor was supported under model M0 as the maximum-likelihood scenario for a high percentage of trees (92%). On the contrary, a widespread western Mediterranean ancestor was supported under model M3 for a similar percentage of trees (93%). Models M1 and M2 yielded a higher uncertainty (see Fig. 4).

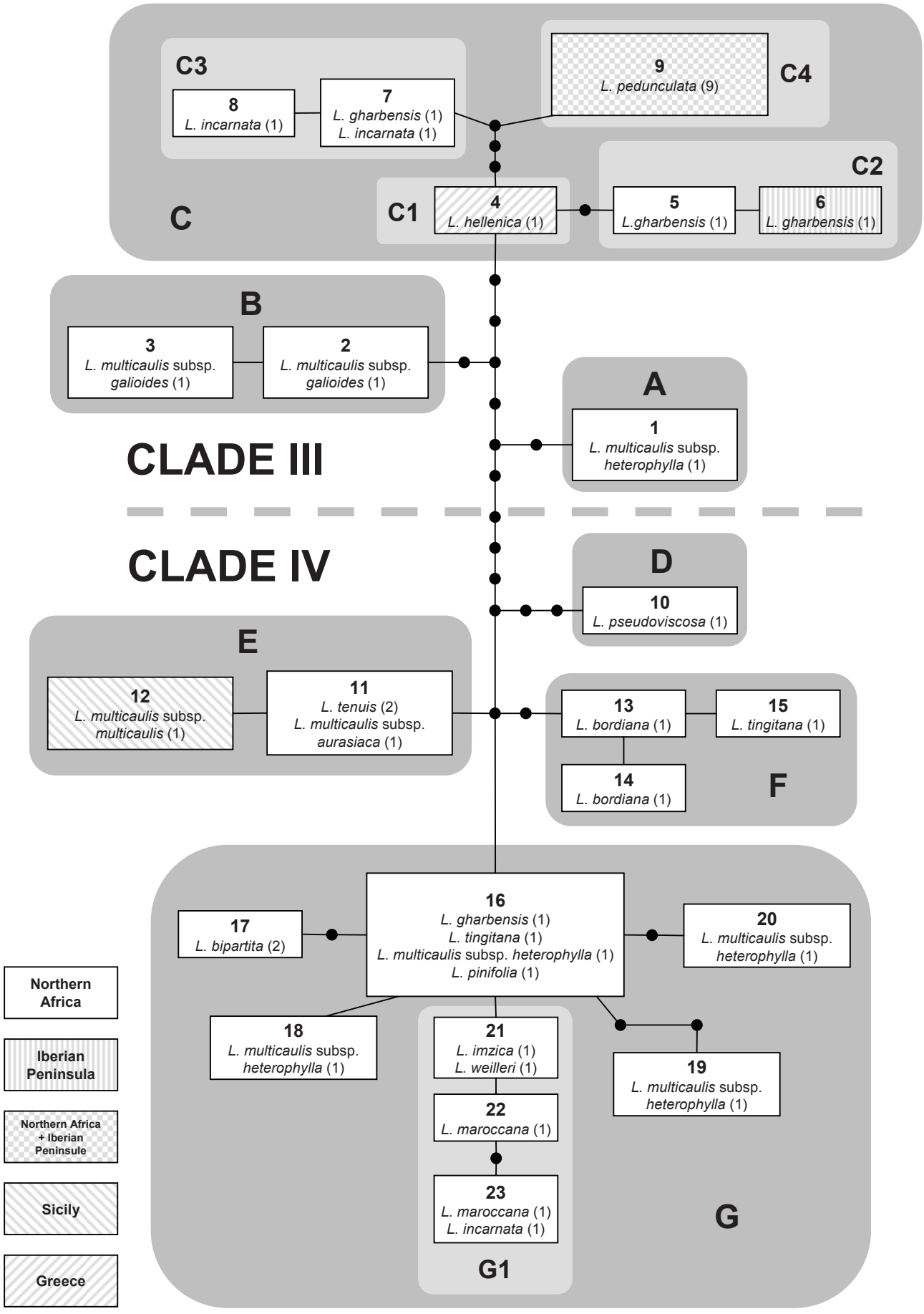
Results of S-DIVA analyses were mostly congruent with DEC inferences. A higher resolution was also found when the maximum number of areas was set to two (Fig. 5). As in DEC

reconstructions, there was uncertainty on the range of the root node (I or AI). An Iberian ancestor was strongly supported under calculations of ancestral probabilities following Harris & Xiang (2009). However, an Iberian and a widespread western Mediterranean ancestor were equally supported under the method of Nylander *et al.* (2008). In all other nodes, the same ancestral ranges were obtained in the four S-DIVA analyses (results not shown), which fit the scenarios inferred by DEC modelling as shown in Fig. 3A.

Haplotype network

The haplotype network analysis of the primarily African lineages (clades III, IV) (Fig. 6) distinguished seven main haplotype lineages (A-G) with a high number (16) of internal missing haplotypes separating them. Haplotypes depicted in Fig. 6 correspond to distinct sequences of dating and biogeographic analyses (Figs. 2, 3 and 5). The geographic distribution of haplotype lineages and sublineages is illustrated in Fig. 7. Only two of these lineages (C and E) were distributed on both Europe and northern Africa. Lineage E included accessions of *L. tenuis* and *L. multicaulis* subsp. *aurasiaca* from central northern Africa together with the accession of *L. multicaulis* subsp. *multicaulis* from Sicily (tip haplotype 12). Lineage C contained similar numbers of European and African samples. Interestingly, a Balkan-African-Iberian connection was obtained because the internal haplotype (4) in lineage C was found in the Greek *L. hellenica*, and was connected to a Moroccan sample of *L. gharbensis* (Haplotype 5), and then to the tip haplotype (6) of the Iberian accession of *L. gharbensis* (see sublineage C2 in Figs. 6 and 7). All nine samples of *L. pedunculata* (five Iberian and four northern African) yielded the same widely-distributed tip haplotype (9, sublineage C4 in Figs. 6 and 7).

Fig. 6. Statistical parsimony network of cpDNA haplotypes (indicated as numbered squares) of primarily northern African clades III and IV. Lines represent single nucleotide substitutions; dots indicate absent haplotypes (extinct or not found). Taxa harbouring each haplotype are shown within the squares, with the number of sequenced individuals indicated in brackets. Geographic distribution of haplotypes is shown, and main clades and lineages mentioned in the text are delimited.


C3
8
L. incarnata (1)
7
L. gharbensis (1)
L. incarnata (1)
9
L. pedunculata (9)
C4
C2
C
C1
4
L. hellenica (1)
5
L.gharbensis (1)
6
L. gharbensis (1)
B
3
L. multicaulis subsp. galioides (1)
2
L. multicaulis subsp. galioides (1)
A
1
L. multicaulis subsp. heterophylla (1)
CLADE III
CLADE IV
D
10
L. pseudoviscosa (1)
E
12
L. multicaulis subsp. multicaulis (1)
11
L. tenuis (2)
L. multicaulis subsp. aurasiaca (1)
13
L. bordiana (1)
15
L. tingitana (1)
14
L. bordiana (1)
F
16
L. gharbensis (1)
L. tingitana (1)
L. multicaulis subsp. heterophylla (1)
L. pinifolia (1)
17
L. bipartita (2)
20
L. multicaulis subsp. heterophylla (1)
18
L. multicaulis subsp. heterophylla (1)
21
L. imzica (1)
L. weilleri (1)
19
L. multicaulis subsp. heterophylla (1)
22
L. maroccana (1)
23
L. maroccana (1)
L. incarnata (1)
G1
G
Northern Africa
Iberian Peninsula
Northern Africa + Iberian Peninsule
Sicily
Greece

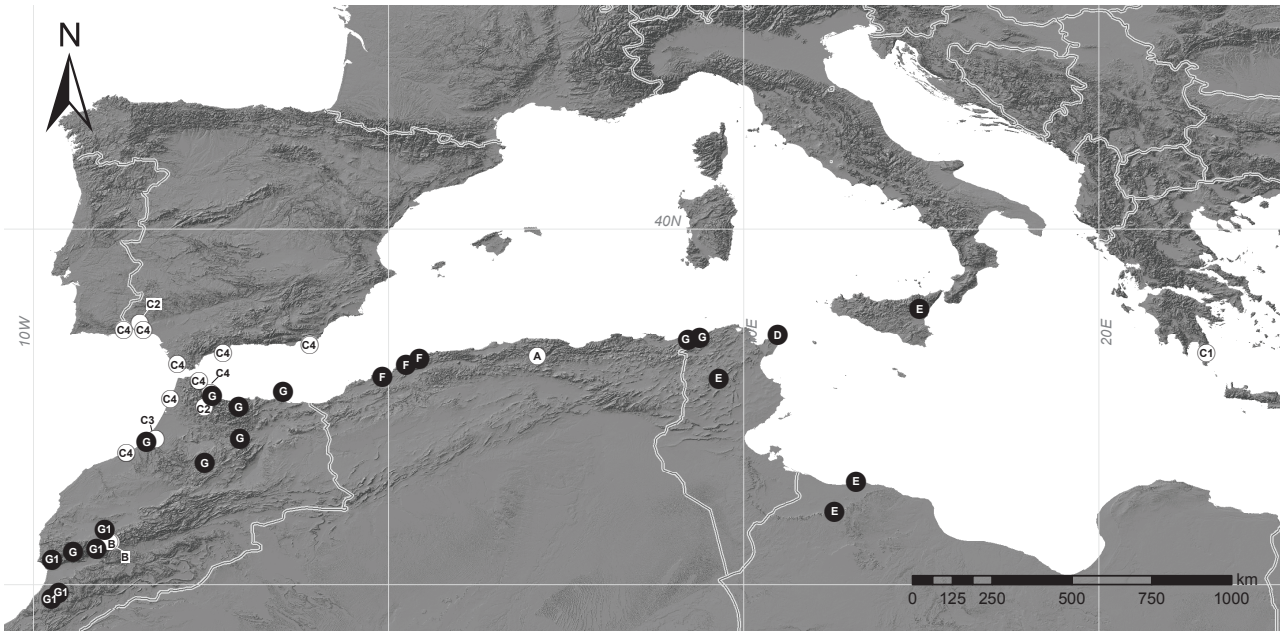


Fig. 7. Geographic distribution of cpDNA haplotype lineages of clades III and IV. Lineages are named as in Fig. 6. Samples of clade III are shown with white circles, while samples of clade IV are shown with black circles.

A lack of agreement between haplotype lineages and species delimitation in northern Africa was apparent for some taxa, specially the widely-distributed and morphologically variable *L. multicaulis*. Samples of different subspecies of *L. multicaulis* were included in four separate haplotype lineages, whereas one of its haplotypes (16) was shared by three other species and had a wide distribution from Morocco to Tunisia. By contrast, taxa endemic to narrower areas (including subspecies of *L. multicaulis*) frequently belonged to equally narrowly distributed haplotype lineages: B (High Atlas; *L. multicaulis* subsp. *galioides*), E (central northern Africa and Sicily; *L. multicaulis* subsp. *multicaulis*, *L. multicaulis* subsp. *aurasiaca*), F (NW Algeria; *L. bordiana*); and G1 (Atlas and Anti-Atlas mountains; *L. maroccana*, *L. imzica*, *L. weilleri*). Incongruences between taxonomy, geography and phylogenetic results were also observed in the phylogenetic analyses (Fig. 1).

Discussion

Complex taxonomy and reliability of biogeographic reconstruction

In our biogeographic analyses, we explicitly designed a morphological and geographic sample to overcome methodological issues associated with the complex taxonomy of *Linaria*. Biogeographic reconstructions are often performed on the basis of a gene tree (or a tree resulting from concatenation of several DNA regions) including a single individual to represent the entire distribution of each species. This is appropriate when the major biogeographic patterns of a large lineage are the focus (Roquet *et al.*, 2009; Buerki *et al.*, 2011; Valente *et al.*, 2011), provided that populations of each species constitute a natural group. When inferring colonization events at a finer scale, as in our case, this approach is more problematic. Assuming naturalness of species and equalling the obtained tree to the species tree may lead to spurious reconstructions under a scenario of rapid, convergent or paralell evolution. Indeed, the prolific diversification of subsect. *Versicolores*, possibly accompanied of incomplete sorting of ancestral polymorphisms and hybridization, accounts for the frequent non-monophyly of con-specific samples of our phylogeny (Joly *et al.*, 2009) (see Fig. 1). In addition, species delimitation is particularly difficult in *Linaria* due to the poor knowledge of some taxa (Viano, 1978a, b; Sutton, 1988). Under this complex scenario, we consider our cpDNA phylogeny and lineage-based area reconstruction the most appropriate approach to circumvent methodological problems to infer colonization patterns. For instance, this approach has revealed that *L. incarnata* might be a polyphyletic taxon. Iberian and Moroccan populations are currently recognized as the same species (Viano, 1969; Sutton, 1988; Valdés *et al.*, 2002; Sáez & Bernal, 2009), yet some morphological differences have been suggested (Sutton, 1988). Our analyses separate them in primarily Iberian or African clades (II and III-IV), and the similar morphologies are more easily explained by parallel evolution than by intercontinental dispersal. Likewise, there is uncertainty on the taxonomic status of Greek populations named as *L. hellenica* by some authors (Contandriopoulos & Yannitsaros, 1975; Sutton, 1988) or assigned to *L. tenuis* by others (Tan & Iatrou, 2001). Our analyses clearly place *L. hellenica* and African samples of *L. tenuis* in separate lineages. In the case of the polyphyletic *L. gharbensis*, Iberian populations were formerly described as *L. heterophylla* subsp. *tartessiana* (Vicioso, 1946) or *L. tartessiana* (Valdés, 1986),

but have only been assigned to *L. gharbensis* in the last treatments (Sánchez-Gullón *et al.*, 2006; Sáez & Bernal, 2009). In this case, a close phylogeographic relationship between the Iberian and one of the African samples of *L. gharbensis* was obtained in our analyses (lineage C2 in Fig. 6), but the species was nevertheless retrieved as polyphyletic.

Miocene origin of the bifid toadflaxes

Given that section *Versicolores* was strongly supported as a monophyletic group by our phylogenetic analysis, the origin and colonization of its lineages can be reliably inferred. In addition, naturalness of sect. *Versicolores* is supported by a diagnostic morphological synapomorphy (discrete stigmatic areas), which is unique in a genus of over 150 species, as already stated by Sutton (1988). The first dichotomy within the group agrees with the infrasectional taxonomy established in Sutton (1988), with subsections *Elegantes* and *Versicolores* being well supported as monophyletic sister groups.

The crown-age of sect. *Versicolores* based on our dating analysis supported an origin of the group around the Middle to Late Miocene. The same might be true for several major lineages of *Linaria* used as the outgroup, as shown by the chronogram in Fig. 2. Increasing of aridity in the Mediterranean area during the Cenozoic culminated in the Messinian Salinity Crisis (Bocquet *et al.*, 1978) and the establishment of the Mediterranean climate (Suc, 1984), and these climatic changes had a major impact in the western Mediterranean flora (Postigo Mijarra *et al.*, 2009). Assuming an herbaceous (annual), xeromorphic ancestor (as most species of sect. *Versicolores*, including those of the basal-most lineages), we interpret specialization of bifid toadflaxes to xeric environments to be associated with climate changes of the Miocene. As species diversification in sect. *Versicolores* (primarily affecting subsect. *Versicolores*) mostly happened during the Pliocene and Quaternary, specialization to annuality and other xerophytic characteristics may have been retained in the bifid toadflaxes since the Miocene.

Historical Europe-Africa isolation

Four major lineages of sect. *Versicolores* (I, II, III, IV) were found in our phylogenetic analysis, which are primarily distributed in the Iberian Peninsula or northern Africa. Our biogeographic reconstructions were not conclusive on the ancestral area of sect. *Versicolores*, except for the statistical DIVA analysis performed following Harris & Xiang (2009), which clearly supported an Iberian ancestor. Regardless of the uncertainty, we are favouring the hypothesis of an Iberian ancestor (Fig. 3B) over a widespread (Iberian-African) ancestor considering the fact that both DEC and DIVA have a tendency to infer widespread ancestors (Ree *et al.*, 2005; Lamm & Redelings, 2009; Kodandaramaiah, 2010). If this is the case, a dispersal event from the Iberian Peninsula to northern Africa would have occurred along the branch leading to subsect. *Versicolores*, which is congruent with a range expansion during the Messinian connection between Europe and Africa (Fig. 3C). Alternative hypotheses are plausible, and a better-supported inference for the ancestral area of sect. *Versicolores* will probably be obtained when the sister group to sect. *Versicolores* is determined.

Despite uncertainty at the root, it is clearly inferred that the Mediterranean Sea acted as an effective sea barrier for colonization by the wingless seeds of *Linaria* sect. *Versicolores* after the refilling of the basin at the Miocene-Pliocene boundary (5.33 Ma). Subsect. *Elegantes* (lineage I) inherited an Iberian ancestral range from the common ancestor, and subsequently gave rise to the two extant Iberian species (*L. elegans* and *L. nigricans*). A widespread Iberian-northern African ancestor of subsect. *Versicolores* was inferred. The well-supported subdivision of its range between Iberian (clade II) and northern African (clades III and IV) sister groups is congruent with lineage isolation that followed the opening of the Strait of Gibraltar in the early Pliocene (Fig. 3D). Geographic isolation between African and European populations and taxa has previously been described (Lumaret *et al.*, 2002; Lumaret *et al.*, 2005; Terrab *et al.*, 2007; Escudero *et al.*, 2008; Font *et al.*, 2009; Jaramillo-Correa *et al.*, 2010). In *Linaria* sect. *Versicolores*, the possibility remains that Pliocene colonizations between Africa and Europe occurred, but the resulting genetic footprint was erased. New connections between extant lineages of both areas have occurred only in recent times, as discussed below.

Recent colonizations over the Mediterranean Sea

The disjunct distribution of any taxa in two areas currently separated by a dispersal barrier may be explained by two alternative scenarios: colonization before barrier formation or dispersal after barrier formation. Distinguishing between these two hypotheses constitutes one of the most discussed topics in historical biogeography (Ronquist, 1997; Sanmartín & Ronquist, 2004; Queiroz, 2005). The widely-used dispersal-vicariance analysis (Ronquist, 1997) does not *a priori* take into account the relative timing of lineage divergence and barrier formation, and therefore is not ideally suited for this task (Kodandaramaiah, 2010). Instead, a combination of time-calibrated phylogenetic data with geologic and paleogeographic information can provide robust inferences, and this is the approach taken by modern analytical methods (Ree *et al.*, 2005; Ree & Smith, 2008) and employed in our study.

Indeed, the well-known paleogeographic framework of the Mediterranean basin (e.g. Jolivet *et al.*, 2006) allows for successful implementation of this approach to analyze the biogeographic history of taxa in this area (Oberprieler, 2005; Mansion *et al.*, 2008; Guzmán & Vargas, 2009; Mansion *et al.*, 2009; Fernández-Mazuecos & Vargas, 2010; Salvo *et al.*, 2010). Species level (phylogenetic) and population level (phylogeographic) analyses have tested disjunct distributions in southern Europe and northern Africa (Burban & Petit, 2003; Lumaret *et al.*, 2005; Petit *et al.*, 2005; Rodríguez-Sánchez *et al.*, 2009). However, only a few studies are placed in a time-calibrated framework and are thus able to date colonization events in relation to the last land connection between both continents during the Messinian. For example, colonization along a Messinian land bridge between Europe and Africa has been proposed for the xerophytic species *Androcymbium gramineum* (Caujapé-Castells & Jansen, 2003) and the *Campanula broussonetiana* / *C. transtagana* lineage (Cano-Maqueda *et al.*, 2008). On the other hand, post-Messinian long-distance colonization over the Mediterranean sea has been strongly supported in several species of *Cistus*, a typically Mediterranean genus (Guzmán & Vargas, 2009; Fernández-Mazuecos & Vargas, 2010). Like *Cistus*, *Linaria* sect. *Versicolores* diversified mainly after the establishment of the Mediterranean climate and underwent events of post-Messinian intercontinental colonization despite lacking any special mechanism for long-distance dispersal. At least four colonization events from Africa to Europe in clades III and IV are supported by our biogeographic reconstructions (Fig. 3 and 5), and the relaxed molecular-clock analysis (Fig.

2) unambiguously places all these events in the last one million years (Quaternary, Fig. 3E). The recent expansion of open Mediterranean habitats may have favoured intercontinental colonization after long-distance dispersal in recent times (Rodríguez-Sánchez *et al.*, 2008).

Intercontinental colonization may have been more likely in those regions where African and European land masses have been closer, particularly the straits of Gibraltar and Sicily. Over the Strait of Gibraltar area, at least two events of jump dispersal are inferred (lineages C2 and C4 of clade III). Recent studies indicate that the Strait of Gibraltar may not have constituted such an important barrier for plant colonization as previously thought (reviewed in Rodríguez-Sánchez *et al.*, 2008). Several events of recent colonization between Africa and Europe in this area have been documented within each of four *Cistus* species (Guzmán & Vargas, 2009; Fernández-Mazuecos & Vargas, 2010) and also in *Linaria* sect. *Supinae* (J.L. Blanco-Pastor & P. Vargas, unpublished). In our case, in lineage C2, the restricted populations of *L. gharbensis* in SW Spain (Sáez & Bernal, 2009) are closely related to populations of the same species in northern Morocco (Figs. 6 and 7), while in lineage C4, all nine samples of *L. pedunculata* (five Iberian and four northern African) shared a common and exclusive cpDNA haplotype (Figs. 6 and 7). This result indicates a recent and rapid expansion of *L. pedunculata* across maritime dunes and sandy beaches of Southern Iberia and NW Africa, which may have been facilitated by the autocompatibility of this species (Docherty, 1982). Marine dispersal may also have played a role, as was the case for other coastal species of the Mediterranean area (Escudero *et al.*, 2010).

Over the Strait of Sicily, our results support a recent colonization by lineage E (clade IV): the tip haplotype 12, found in Sicily (*L. multicaulis* subsp. *multicaulis*), is closely related to haplotype 11, found in three samples from Tunisia and Lybia (*L. multicaulis* subsp. *aurasiaca* and *L. tenuis*) (Figs. 6 and 7). Similarly to the Strait of Gibraltar, the Strait of Sicily was formed with the refilling of the Mediterranean at the beginning of the Pliocene, which flooded the Messinian landbridge that last connected northern Africa and the landmass that later became Sicily (Figs. 3C and 3D) (Thiede, 1978; Stöck *et al.*, 2008). Although the Strait is now 140 km wide, it may have been reduced to c. 50 km during Pleistocene glaciations, which does not affect our hypothesis of jump dispersal from northern Africa to Sicily across a marine sea barrier. Long-distance dispersal over the Strait of Sicily has also been documented for the *Anthemis secundiramea*

group (Lo Presti & Oberprieler, 2009, 2011) and for *Cistus salviifolius* (Fernández-Mazuecos & Vargas, 2010).

The fourth inferred event of Africa-Europe colonization is harder to interpret. It is shown by the presence of haplotype 4 in Greek populations of the endangered *L. hellenica* (Contandriopoulos & Yannitsaros, 1975; Sutton, 1988). Our analyses placed this haplotype in a lineage mostly including samples from the area surrounding the Strait of Gibraltar (lineage C; Figs. 6 and 7), and very unconnected to the morphologically and geographically related samples of *L. tenuis*. Although our data clearly support long-distance dispersal from northern Africa, a deeper sampling, particularly in NE Africa, is needed to confirm whether the Greek populations are indeed the result of colonization from the Strait of Gibraltar area. In any case, long-distance colonization between the western and eastern Mediterranean has been suggested for other taxa, such as the coastal *Calystegia soldanella* (Arafeh & Kadereit, 2006), and again the widely-distributed Mediterranean shrub *Cistus salviifolius* (Fernández-Mazuecos & Vargas, 2010).

In summary, the causes behind the disjunct distribution of bifid toadflaxes in the Iberian Peninsula and northern Africa have been carefully addressed in a time-calibrated phylogenetic framework. The Mediterranean Sea acted as a relatively effective barrier for lineage connections of *Linaria* sect. *Versicolores* since the end of the Miocene. In fact, Iberian and northern African lineages appear to have diversified in isolation after the Pliocene refilling of the basin. However, some colonization events from northern Africa to Europe in very recent times (< 1 Ma) are clearly attributable to intercontinental colonization, despite the absence of specific mechanisms for long-distance dispersal. The small size of the seeds and the abundance of open and sandy habitats in the Mediterranean region probably favoured these events. Therefore, processes of both geographic isolation and long-distance dispersal may have taken place and explain the current distribution of *Linaria* sect. *Versicolores* lineages across the Mediterranean basin.

Acknowledgements

We thank Emilio Cano, Fátima Durán and Gemma Andreu for laboratory assistance; J.J. Aldasoro, E. Amat, B. Estébanez, F. Gómiz, B. Guzmán, S. Martín-Bravo, E. Rico, the *Flora iberica* project, the

MA, ATH, UPOS and SALA herbaria, and particularly the RNG herbarium and its curator S.L. Jury for plant material; J. Ramírez, E. Sánchez-Gullón, J.C. Moreno, J.L. Blanco and D. Orgaz for field assistance; the "Marismas del Odiel" natural reserve for collection permissions; L. Valente and J. L. Blanco for critical reading and comments that improved the quality of the manuscript.

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Supporting Information

Table S1. Voucher specimens and GenBank accession numbers of sampled taxa and populations of *Linaria* sect. *Versicolores* and the outgroup. Sequence / haplotype codes are shown for ingroup samples (as in Figs. 2, 3, 5 and 6).

Taxon	(Population No.) Sampled locality	Voucher	Sequence / haplotype code	*rpl*32-*trn*L^{UAG} accession no.	*trn*K-*mat*K accession no.
Antirrhinum L.					
Antirrhinum graniticum Rothm.	Spain, Cáceres, Trujillo	P. Vargas 213PV06 (MA)	-	JF694120	JF694188
Chaenorhinum (DC.) Rchb.					
Chaenorhinum macropodum (Boiss. & Reut.) Lange	Spain, Málaga, Cómpeta	P. Vargas 27PV08 (MA)	-	JF694119	JF694187
Linaria Mill.					
Linaria sect. *Diffusae* (Benth.) Wettst.					
L. reflexa (L.) Chaz.	Algeria, Algiers	J.J. Aldasoro A9799 (MA)	-	JF694129	JF694197
Linaria sect. *Linaria*					
L. vulgaris Mill.	France, Chamonix	B. Estébanez s.n. (MA)	-	JF694126	JF694194
Linaria sect. *Macrocentrum* D.A.Sutton					
L. chalepensis (L.) Mill.	Cyprus, Larnaca, Cape Kiti	Iter Mediterraneum IV 294 (MA)	-	JF694127	JF694195
Linaria sect. *Pelisserianae* Valdés					
L. triornithophora (L.) Willd.	Spain, Cáceres, Puerto de Perales	M. Fernández-Mazuecos 18MF07 (MA)	-	JF694128	JF694196
Linaria sect. *Speciosae* (Benth.) Wettst.					
L. genistifolia (L.) Mill.	Turkey, Hadim-Bezkir	J.J. Aldasoro & M.L. Alarcón A9751 (MA)	-	JF694124	JF694192
L. repens (L.) Mill.	Spain, Cuencia, Beteta	M. Fernández-Mazuecos 54MF09 (MA)	-	JF694125	JF694193
Linaria sect. *Supinae* (Benth.) Wettst.					
L. alpina (L.) Mill.	Spain, Huesca, Bujaruelo	J. Güemes s.n. (MA)	-	JF694121	JF694189
L. amoi Campo ex Amo	Spain, Málaga, Cómpeta	M. Fernández-Mazuecos, A.D. Forrest & P. Vargas 30PV08 (MA)	-	JF694122	JF694190
L. micrantha (Cav.) Hoffmanns. & Link	Spain, Alicante, Vall de Gallinera	J.X. Soler & M. Signes 1530-JXS (MA)	-	JF694123	JF694191
Linaria sect. *Versicolores* (Benth.) Wettst.					
Subsect. *Versicolores*					
L. algarviana Chav.	Portugal, Cabo de São Vicente	M. Fernández-Mazuecos 11MF09 (MA)	Ib6	JF694130	JF694198
L. bipartita (Vent.) Willd.	(1) Morocco, Rabat	S.L. Jury with R.G. Wilson 18558 (RNG)	17	JF694131	JF694199
	(2) Morocco, Tamri	S.L. Jury, B. Tahmi & T.M. Upson 14293 (RNG)	17	JF694132	JF694200
L. bordiana Santa & Simonneau	(1) Algeria, Mostaganem – Tenes	Davis 51833 (RNG)	13	JF694134	JF694202
	(2) Algeria, Sidi Lakhdar	D.A. & S.J. Sutton 172 (RNG)	14	JF694133	JF694201
L. clementei Haensel. ex Boiss.	(1) Spain, Málaga, Alhaurín de la Torre	M. Fernández-Mazuecos, A.D. Forrest & P. Vargas 7MF08 (MA)	Ib2	JF694135	JF694203
	(2) Spain, Málaga, Coín	M. Fernández-Mazuecos & J. Ramírez 24MF09 (MA)	Ib1	JF694136	JF694204
L. gharbensis Batt. & Pit.	(1) Morocco, Bou Ahmed	S.L. Jury, A. Taleb, T.M. Upson & G.S Waltens 13406 (RNG)	16	JF694137	JF694205
	(2) Morocco, Chefchaouen	J. Montserrat & J. Vicens JMM-4193/5 (RNG)	5	JF694177	JF694245
	(3) Morocco, Rabat	S.L. Jury & R.G. Wilson 18559 (RNG)	7	JF694138	JF694206
	(4) Spain, Huelva, Gibraleón	M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 7MF09 (MA)	6	JF694139	JF694207
L. hellenica Turrill	Greece, Kambos	Unknown collector (ATH)	4	JF694140	JF694208
L. imzica Gómiz	Morocco, Jbel Imzi	F. Gómiz s.n. (MA)	21	JF694141	JF694209

Table S1. Continued.

L. incarnata (Vent.) Spreng.	(1) Morocco, Kenitra-Khemisset	S. Martín-Bravo, I. Pulgar, F.J. Fernández, G.C. Mazo 34SMB06 (MA)	8	JF694142	JF694210
	(2) Morocco, Marrakech	S.L. Jury 14151 (RNG)	23	JF694143	JF694211
	(3) Morocco, Salé	J. Lambinon & G. van den Sande nº95/Ma/333 (RNG)	7	JF694144	JF694212
	(4) Spain, Badajoz, Alburquerque	M. Fernández-Mazuecos 9MF09 (MA)	Ib6	JF694145	JF694213
	(5) Spain, Salamanca, Pelabravo	M. Fernández-Mazuecos & P. Vargas 39MF09 (MA)	Ib7	JF694146	JF694214
	(6) Spain, Huelva, Valverde del Camino (= *L. onubensis* Pau)	V. Valcarcel & P. Vargas 5PV08 (MA)	Ib6	JF694147	JF694215
L. maroccana Hook.f.	(1) Morocco, Imouzzer Valley	M. Ait Lafkih s.n. (RNG)	23	JF694148	JF694216
	(2) Morocco, Marrakech – Tizi-n-Test	S.L. Jury, B. Tahiri & T.M. Upson 14209 (RNG)	22	JF694149	JF694217
L. multicaulis (L.) Mill.					
subsp. *multicaulis*	Italy, Sicily, Etna	I. Álvarez *et al.* IA1622 (MA)	12	JF694150	JF694218
subsp. *aurasiaca* (Pomel) D.A.Sutton	Tunisia, El Kesra	Davis & Lamond 57154 (RNG)	11	JF694151	JF694219
subsp. *galioides* (Ball) D.A.Sutton	(1) Morocco, Oukaimedem	P. Jiménez Mejías, E. Narbona, A.J. Chaparro & M. Parra 200PJM05 (UPOS)	2	JF694152	JF694220
	(2) Morocco, Yagour	A. Kool with H.J. Boer, P. Domínguez 904 (RNG)	3	JF694153	JF694221
subsp. *heterophylla* (Desf.) D.A.Sutton	(1) Algeria, Tikjda	Davis 53078 (RNG)	1	JF694154	JF694222
	(2) Morocco, Azrou	M. Fernández-Mazuecos & J.C. Moreno 15MF08 (MA)	20	JF694155	JF694223
	(3) Morocco, Beni-Hadifa	B. Guzmán 108bBGA04 (MA)	18	JF694156	JF694224
	(4) Morocco, Jbel Tazekka	E. Rico, S. Andrés & M. Santos SA-221 (SALA)	19	JF694157	JF694225
	(5) Tunisia, Tabarka	J.J. Aldasoro A2888 (MA)	16	JF694158	JF694226
L. pedunculata (L.) Chaz.	(1) Morocco, Chefchaouen	M.A. Mateos, M.C. Reina, G. Sangalli, N. Sardón & B. Valdés s.n. (RNG)	9	JF694163	JF694231
	(2) Morocco, Larache	Silvestre, G.-Rowe & Vilches s.n. (RNG)	9	JF694167	JF694235
	(3) Morocco, Mdiq	W. Lippert 24229 (RNG)	9	JF694164	JF694232
	(4) Morocco, Mohammedia	Aparicio, Rowe & Silvestre s.n. (RNG)	9	JF694165	JF694233
	(5) Portugal, Monte Gordo	M. Fernández-Mazuecos & J.L. Blanco 61MF10 (MA)	9	JF694166	JF694234
	(6) Spain, Almería, Cabo de Gata	M. Fernández-Mazuecos 30MF09 (MA)	9	JF694159	JF694227
	(7) Spain. Cádiz, Barbate	E. Sánchez-Gullón s.n. (MA)	9	JF694160	JF694228
	(8) Spain, Huelva, Marismas del Odiel	M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 4MF09 (MA)	9	JF694161	JF694229
	(9) Spain, Málaga, Mijas	M. Fernández-Mazuecos & J. Ramírez 27MF09 (MA)	9	JF694162	JF694230
L. pinifolia (Poir.) Thell.	Tunisia, El Kala	A. Dubuis, H. Maurel & R. Rhamoun s.n. (RNG)	16	JF694168	JF694236
L. pseudoviscosa Murb.	Tunisia, El Haouaria	P. Wilkin & E.J. Wellens 231 (RNG)	10	JF694169	JF694237
L. salzmannii Boiss.	Spain, Málaga, El Chorro	M. Fernández-Mazuecos & J. Ramírez 19MF09 (MA)	Ib1	JF694170	JF694238
L. spartea (L.) Chaz.	(1) Spain, Madrid, Colmenar	P. Vargas 101PV07 (MA)	Ib6	JF694171	JF694239
	(2) Spain, Soria, Tardelcuende	M. Fernández-Mazuecos, A. Quiroga, S.C. Herrera & D. Orgaz 14MF07 (MA)	Ib3	JF694172	JF694240
L. tenuis (Viv.) Spreng.	(1) Libya, Tripoli	Davis & Boulos 50581 (RNG)	11	JF694174	JF694242
	(2) Libya, Gebel Nefoussa	Davis 49632 (RNG)	11	JF694173	JF694241
L. tingitana Boiss. & Reut.	(1) Algeria, El Macta	D.A. & S.J. Sutton 383 (RNG)	15	JF694175	JF694243
	(2) Morocco, Cap des Trois Fourches	T.M. Upson and Ait Lafkih, M. Hassan, G.S. Walters 14012 (RNG)	16	JF694176	JF694244
L. viscosa (L.) Chaz.					
subsp. *viscosa*	(1) Spain, Huelva, Marismas del Odiel	M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 6MF09 (MA)	Ib4	JF694178	JF694246
	(2) Spain, Huelva, Matalascañas	M. Fernández-Mazuecos & J.L. Blanco 1MF09 (MA)	Ib5	JF694179	JF694247
subsp. *spicata* (Coutinho) D.A.Sutton	(1) Spain, Jaén, Cazorla	M. Fernández-Mazuecos 49MF09 (MA)	Ib1	JF694181	JF694249
	(2) Spain, Málaga, Cómpeta	M. Fernández-Mazuecos, A.D. Forrest & P. Vargas 9MF08 (MA)	Ib1	JF694180	JF694248
L. weilleri Emb. & Maire	Morocco, Tirhmi	Miller, Russell & Sutton s.n. (RNG)	21	JF694182	JF694250
Subsect. Elegantes (Viano) D.A.Sutton					
L. elegans Cav.	(1) Spain, Orense, San Xoán de Río	M. Fernández-Mazuecos 45MF08 (MA)	Le2	JF694184	JF694252
	(2) Spain, Ávila, Plataforma de Gredos	E. Amat s.n. (MA)	Le1	JF694183	JF694251
L. nigricans Lange	(1) Spain, Almería, Tabernas	P. Vargas 3PV08 (MA)	Ln2	JF694186	JF694254
	(2) Spain, Almería, Cabo de Gata	M. Fernández-Mazuecos 29MF09 (MA)	Ln1	JF694185	JF694253

Capítulo 4

Evolución de la forma floral en *Linaria* sect. *Versicolores*: caracteres restrictivos para los polinizadores determinan óptimos evolutivos con tasas de diversificación contrastadas

Pollinator-restrictive flower traits determine evolutionary optima with differential diversification rates in bifid toadflaxes (*Linaria* sect. *Versicolores*)

Este capítulo se ha desarrollado en colaboración con José María Gómez (Universidad de Granada) y José Luis Blanco Pastor (Real Jardín Botánico, CSIC).

Manuscrito inédito

Abstract

The role of flower specialization in plant speciation and evolution remains controversial. Here we analyzed the evolution of flower traits restricting the access to pollinators in the bifid toadflaxes (*Linaria* sect. *Versicolores*), a monophyletic group of c. 30 species and subspecies with highly specialized corollas. A time-calibrated phylogeny based on both nuclear and plastid DNA sequences was obtained using a coalescent-based method, and flower morphology was characterized by means of morphometric analyses. Using recently-developed methods, directional trends in morphological traits and trait-dependent diversification rates were jointly analyzed, and morphological shifts were reconstructed along the phylogeny. Our results indicated that a restrictive character state (narrow corolla tube) may be ancestral to *Linaria* sect. *Versicolores*. After its early loss in the most species-rich clade, this character state has been convergently reacquired in multiple lineages of this clade in recent times, yet it has exerted a negative influence on diversification rates. Pollinator surveys and comparative analyses suggest that the narrow- and broad-tubed flowers are evolutionary optima representing divergent strategies of pollen placement on nectar-feeding insects. We therefore suggest that opposing individual-level and species-level selection pressures may have driven the evolution of pollinator-restrictive traits in bifid toadflaxes.

Introduction

Variation of flower morphological traits has long been considered to drive evolution and diversification of angiosperms (Darwin, 1862, 1877; Grant, 1949; Stebbins, 1970; Kay & Sargent, 2009; van der Niet & Johnson, 2012). Adaptation to different pollinator vectors (particularly animal pollinators) has been hypothesized to be a major force modelling flower morphology. This notion gave rise to the concept of pollination syndromes, i.e. sets of flower traits (shape, colour, nectar, scent) that have convergently evolved in distant plant lineages as an adaptation to particular pollinators (bees, birds, moths, etc.) (Faegri & van der Pijl, 1979; Fenster *et al.*, 2004). The concept of pollination syndromes, which relies on flower specialization, has been challenged by several studies that emphasize the prevalence of generalist flowers in nature and the low power of pollination syndromes as predictors of actual pollinators (Waser *et al.*, 1996; Ollerton *et al.*, 2009). Indeed, plant lineages in which flower evolution can be described in terms of pollinator shifts associated to changes between ideal syndromes seem to be rare (e.g. Wilson *et al.*, 2006; Whittall & Hodges, 2007). Specialization may still play a relevant role in plant speciation (Kay & Sargent, 2009), but syndrome shifts may not account for the majority of speciation events, even in plant lineages in which pollination syndromes are apparent and display extraordinary diversity (Valente *et al.*, 2012). Therefore, instead of focusing on the evolution of syndromes, the investigation of particular traits, including their evolutionary trends, shifts and correlations, is probably more fruitful for the understanding of flower evolution in most plant lineages (Smith, 2010). Of exceptional interest are those traits that restrict the access of pollinators to flower rewards (nectar, pollen), because these physical barriers may have evolved as a specialization to particular pollinators. For example, the evolution of the length of nectar spurs has been the subject of several studies (Herrera, 1990; Johnson & Steiner, 1997; Whittall & Hodges, 2007). Interestingly, Whittall & Hodges (2007) reported a trend towards increasingly long spurs in columbines, linked to directional shifts to pollinators with longer tongues. Variation in length and width of flower tubes provide additional mechanisms for pollinator selection (Alexandersson & Johnson, 2002; Pérez *et al.*, 2004; Tripp & Manos, 2008). An extreme case of restriction to pollinator access is the personate corolla of snapdragons (*Antirrhinum*) and some relatives of the tribe Antirrhineae and the order Lamiales (Sutton, 1988; Endress, 1994; Kampny, 1995). These species display zygomorphic, gamopetalous, bilabiate corollas in which the lower lip is conspicuously arched upwards, constituting a palate.

This structure closes access to the corolla throat, therefore making the mechanical opening of the corolla necessary for insect pollination. The personate corolla has long been considered as an adaptation to bee pollination (mellitophily) (Hill, 1909; Müller, 1929; Sutton, 1988; Endress, 1992; Vargas *et al.*, 2010), as insects other than bees would not be strong or heavy enough to open it (but see Amat *et al.*, 2011).

The relationships between changes in restrictive flower traits and diversification (speciation minus extinction) rates remain poorly understood. Nectar spurs have been hypothesized to represent a key innovation that promotes species diversification by providing a mechanism of pre-zygotic reproductive isolation through differential pollinator visitation (Hodges & Arnold, 1995; Hodges, 1997; but see Hagen & Kadereit, 2003; Cacho *et al.*, 2010). On the other hand, it has been historically argued that ecological specialists usually evolve from generalists, and that specialization constitutes an evolutionary dead-end, i.e. a derived state from which both reversal to a generalist state and shift to a different specialized state would be unlikely (see Futuyma & Moreno, 1988). There are, however, many examples that contradict this idea (see Gómez & Zamora, 2006). In particular, such view has been challenged by phylogeny-based analyses of flower evolution (Tripp & Manos, 2008; Fleming *et al.*, 2009). Simultaneous estimations of rates of character change and state-dependant speciation/extinction rates across phylogenetic trees are crucial for a correct understanding of character evolution (Maddison, 2006; Goldberg & Igić, 2008). Recently-developed methods (Maddison *et al.*, 2007; FitzJohn *et al.*, 2009; FitzJohn, 2010) enable such estimations and hold great promise for understating flower evolution (Smith, 2010), yet they have been rarely applied in this context (but see Armbruster *et al.*, 2009; Smith *et al.*, 2010; Valente *et al.*, 2012).

Toadflaxes (*Linaria* Mill.) constitute the most species-rich (c. 150 species) genus of the snapdragon lineage (tribe Antirrhineae) (Sutton, 1988). *Linaria* pollination has long attracted the interest of botanists and evolutionary biologists (Sprengel, 1793; Darwin, 1876). Toadflaxes constitute a natural group (Vargas *et al.*, 2004; Chapter 2), and display a remarkably diverse array of flower traits whose evolution has not, however, been analyzed to date in a phylogenetic framework. Several traits of *Linaria* flowers are potentially linked to pollinator specialization: they have a zygomorphic, bilabiate, usually personate corolla in which a spur of variable length is formed at the base of the lower lip. The spur contains nectar dripping down from a nectary

located at the base of the ovary (Valdés, 1970). The two pairs of anthers are placed at slightly different heights, with the stigma in the space between (Hill, 1909). While most species have well-developed palates that close access to the corolla throat, in some species belonging to sections *Versicolores*, *Macrocentrum* and *Lectoplectron* the palate is poorly developed, and access to the corolla throat is wide-open. This seems to be usually related to a narrowing of the corolla tube and a broadening of the lower lip (Viano, 1969; Sutton, 1980). Such morphology has been suggested to be related to pollination by long-tongued lepidopterans and dipterans (Hill, 1909; Sutton, 1980, 1988), while the typical personate corolla would be linked to bee pollination (Hill, 1909; Arnold, 1982; Sánchez-Lafuente, 2007), as in *Antirrhinum* (Vargas *et al.*, 2010).

Linaria sect. *Versicolores* (bifid toadflaxes) is an assemblage of c. 25 species mainly distributed in the western Mediterranean region (Sutton, 1988; see examples in Fig. 1). According to phylogenetic analyses based on both nuclear and plastid DNA markers (Chapter 2; Appendix 2) bifid toadflaxes constitute a monophyletic group within *Linaria*, formed by two well-supported sister groups: subsect. *Elegantes* (two species) and subsect. *Versicolores* (c. 23 species). All species are diploid (2n = 12) except for the tetraploid *L. hellenica* (Contandriopoulos & Yannitsaros, 1975; Sutton, 1988), and most species seem to be allogamous (Bruun, 1937; Docherty, 1982; Fernández-Mazuecos & Vargas, unpublished results). Section *Versicolores* constitutes an ideal system for the evolutionary analysis of several flower traits that restrict the access of pollinators to nectar reward: spur length, tube width and palate development. Although morphological affinities among species have not been analyzed in detail, some divergent traits have been described. At least the two species of subsect. *Elegantes* (*L. elegans* and *L. nigricans*) display a widely open corolla with a narrow tube (Viano, 1978b), while species of subsect. *Versicolores* usually exhibit typical personate (closed) corollas with a wider tube. Some authors, however, have suggested that flowers of certain species of subsect. *Versicolores* (*L. incarnata*, *L. bipartita*) resemble those of subsect. *Elegantes* regarding their narrow tubes and broad lower lips (Viano, 1969; Sutton, 1988). In addition, sect. *Versicolores* exhibits a wide variation in spur lengths, including some of the shortest (*L. clementei*) and longest (*L. elegans*) spurs in the genus (Sutton, 1988; Sáez & Bernal, 2009). These traits seem to be associated with contrasting species diversities: only a few species seem to display narrow tubes, and spurs as short as those of *L. clementei* seem to be a rare occurrence. Nevertheless, inter- and

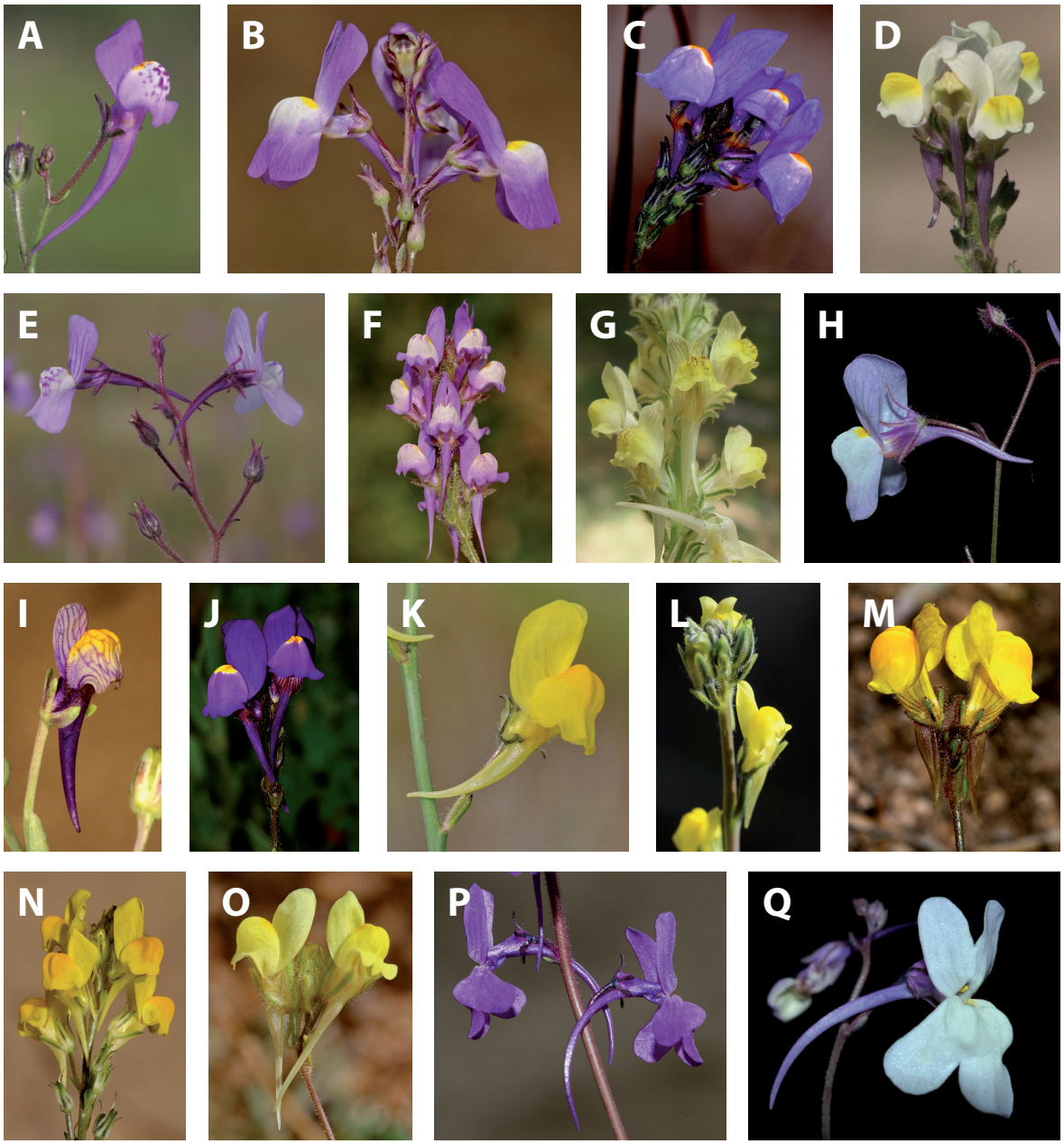


Fig. 1. Representatives of *Linaria* sect. *Versicolores*. Subsect. *Versicolores*: (A) *L. algarviana*; (B) *L. bipartita*; (C) *L. clementei*; (D) *L. gharbensis*; (E) *L. incarnata*; (F) *L. maroccana*; (G) *L. multicaulis* subsp. *heterophylla*; (H) *L. onubensis*; (I) *L. pedunculata*; (J) *L. salzmannii*; (K) *L. spartea*; (L) *L. tenuis*; (M) *L. viscosa* subsp. *spicata*; (N) *L. viscosa* subsp. *viscosa*; (O) *L. weilleri*. Subsect. *Elegantes*: (P) *L. elegans*; (Q) *L. nigricans*. Photos by A. Fernández-Mazuecos (A, E), J. Quiles (B, F, K, N, O), J. Ramírez (C, I, J, M), J.L. Blanco-Pastor (D), E. Rico (G), P. Vargas (H, P, Q) and O. Fragman-Sapir (L).

intraspecific morphological variability has not been quantitatively assessed to date, and the extent to which diversity asymmetries are due to differential rates of diversification (speciation and/or extinction) *vs.* differential rates of change between character states is unknown.

Here we analyzed the evolution of flower morphology in *Linaria* sect. *Versicolores* using phylogenetic and comparative methods to: (1) get a deeper insight into the phylogenetic relationships within this monophyletic group based on both nuclear and plastid DNA sequences; (2) evaluate intra- and interspecific morphological variation of traits determining pollinator access to nectar reward; (3) analyze whether differences in trait-dependent species diversities are due to differential diversification rates *vs.* differential rates of change; and (4) estimate whether the observed patterns of trait evolution may be correlated to pollinator shifts.

Materials and methods

Taxonomic treatment

The potential effects of alpha taxonomy on diversification rate analyses have been remarked by some authors (Marazzi & Sanderson, 2010; Valente *et al.*, 2010a). Indeed, correct species delimitation is crucial to obtain accurate estimates of speciation and extinction rates. We first conducted a review of taxonomic literature and a survey of herbarium specimens mainly from two herbaria with a broad representation of *Linaria* sect. *Versicolores* specimens from Iberia (MA) and northern Africa (RNG) (see Supporting Information Appendix S1). Although the first modern synthesis of the group is due to Viano (Viano, 1978a, b), here we broadly adopted the more inclusive taxonomic treatment of Sutton (1988), except for some modifications as follows (Table 1). First, we have accepted the recently described *L. imzica*, an endemic species from the Moroccan Anti-Atlas with distinct vegetative traits (broad leaves) (Gómiz, 2004). We have also recognized *L. gattefossei*, a Moroccan taxon treated as a synonym of *L. multicaulis* subsp. *heterophylla* by Viano (Viano, 1978a) and Sutton (1988), but subsequently accepted by Fennane & Ibn Tattou (1998). The big leaves and conspicuous whole-plant glandular-pubescence clearly make it a distinct taxon. The Ibero-Moroccan *L. incarnata*, as treated by Sutton (1988), has been disintegrated in three taxa, based on a recent morphological and molecular analysis

Table 1. Taxonomic treatment followed in this chapter, taxon distributions and individuals sampled for phylogenetic and morphometric analyses. Flower morphological type (as discussed in the text) is indicated for each taxon. (1) No. individuals sampled for phylogenetic analyses. (2) No. individuals sampled for spur and tube measures. (3) No. individuals sampled for geometric morphometric analysis. (4) Morphological type.

Taxon	Distribution	(1)	(2)	(3)	(4)
Linaria Mill.					
Linaria sect. *Versicolores* (Benth.) Wettst.					
Subsect. *Versicolores*					
L. algarviana Chav.	SW Portugal (Algarve)	1	7	24	I
L. bipartita (Vent.) Willd.	W Morocco	2	46	-	III
L. bordiana Santa & Simonneau					
subsp. *bordiana*	Algeria	1	8	-	III
subsp. *kralikiana* (Maire) D.A.Sutton	NW Africa	2	4	-	I
L. clementei Haensel. ex Boiss.	S Spain (Málaga)	2	22	21	II
L. dissita Pomel	NW Africa	-	6	-	I
L. gattefossei Maire & Weiller	C Morocco	1	6	-	I
L. gharbensis Batt. & Pit.	NW Africa, SW Spain	2	29	23	I
L. hellenica Turrill	S Greece	1	2	-	I
L. imzica Gómiz	S Morocco (Anti Atlas)	1	9	-	I
L. incarnata (Vent.) Spreng.	W Iberian Peninsula	2	36	48	III
L. mamorensis Mazuecos, Vigalondo & L.Sáez	NW Morocco	2	34	-	III
L. maroccana Hook.f.	Morocco (mainly High Atlas)	2	31	-	I
L. multicaulis (L.) Mill.					
subsp. *multicaulis*	Sicily, S Italy (Calabria)	1	4	-	I
subsp. *aurasiaca* (Pomel) D.A.Sutton	Tunisia, NE Algeria	1	3	-	I
subsp. *galioides* (Ball) D.A.Sutton	Morocco (High Atlas)	2	31	-	I
subsp. *heterophylla* (Desf.) D.A.Sutton	NW Africa	2	46	-	I
L. onubensis Pau	SW Spain (Huelva)	2	15	45	III
L. pedunculata (L.) Chaz.	S Iberian Peninsula, NW Africa, Balearic islands	2	39	27	I
L. pinifolia (Poir.) Thell.	Tunisia, Algeria	1	5	-	I
L. pseudoviscosa Murb.	Tunisia	1	7	-	I
L. salzmannii Boiss.	S Spain (Málaga)	1	5	20	I
L. spartea (L.) Chaz.	Iberian Peninsula, S France	2	87	25	I
L. tenuis (Viv.) Spreng.	N Africa, Middle East	2	3	-	I
L. tingitana Boiss. & Reut.	NW Africa	1	12	-	I
L. viscosa (L.) Chaz.					
subsp. *viscosa*	S Iberian Peninsula	2	60	45	I
subsp. *spicata* (Coutinho) D.A.Sutton	SE Iberian Peninsula	1	45	24	I
L. weilleri Emb. & Maire	S Morocco (Anti Atlas)	1	4	-	I
Subsect. Elegantes (Viano) D.A.Sutton					
L. elegans Cav.	NW Iberian Peninsula	2	58	24	III
L. nigricans Lange	SE Spain (Almería)	2	32	43	III

(Appendix 3): *L. incarnata s.s.*, from Portugal and CW Spain; *L. onubensis*, from SW Spain; and *L. mamorensis*, from Morocco.

In contrast, we have not accepted several other recently described taxa which are poorly differentiated from those of Sutton's (1988) revision. Thus, new subspecific taxa of *L. multicaulis* (Dobignard, 1997; De Leonardis *et al.*, 1999; De Leonardis *et al.*, 2003) have been considered to be referable to those previously described. *L. iranica* (Hamdi *et al.*, 2009) was treated as a synonym of the widespread *L. tenuis*. Regarding the Greek species *L. hellenica*, recognized by Sutton (1988) and Contandriopoulos & Yannitsaros (1975) but assigned to *L. tenuis* by Tan & Iatrou (2001), we have kept it on the basis of its distinct phylogenetic position as inferred by plastid DNA sequences (Chapter 3). Finally, we excluded the Portuguese *L. viscosa* subsp. *crassifolia* (Sutton, 1988) because it was not accepted in the last taxonomic revision of Iberian *Linaria* (Sáez & Bernal, 2009), an is probably referable to *L. viscosa* subsp. *viscosa* or *L. spartea* (L. Sáez, pers. comm.). In the end, we accepted 30 taxa, including species and subspecies (Table 1), which are morphologically and geographically cohesive.

Phylogenetic relationships and divergence times

Sampling strategy and DNA sequencing

We sampled a total of 45 specimens of *Linaria* sect. *Versicolores*, including representatives of 29 of the 30 recognized species and subspecies (one or two specimens per taxon; Table 1; Supporting Information Table S1). To minimize the impact of recent hybridization, we selected unambiguously identified individuals, and some with intermediate traits or uncertain identification were discarded. We only failed to sample *L. dissita*, which is a poorly known northern African taxon (Sutton, 1988; Fennane & Ibn Tattou, 1998). As the outgroup, we sampled nine additional species representing six other sections of *Linaria*, one species of *Chaenorhinum* and one of *Antirrhinum*. Plant material was collected in the field and dried in silica gel or obtained from herbarium collections (RNG, MA, ATH, UPOS; Supporting Information Table S1).

For phylogenetic analyses, we selected one nuclear (ITS) and three plastid (*rpl*32-*trn*L^{UAG}, *trn*K-*mat*K and *trn*S-*trn*G) DNA regions previously employed in phylogenetic and phylogeographic analyses of the genus *Linaria* (Chapter 2; Chapter 3; Blanco-Pastor *et al.*, 2012). Most *rpl*32-*trn*L^{UAG} and *trn*K-*mat*K sequences and some ITS sequences were taken from our previous studies (Chapter 2; Chapter 3). The remaining sequences were newly generated. Procedures used for DNA extraction, amplification and sequencing of DNA regions followed those of Chapters 2 and 3. All sequences were assembled in Geneious Pro (Drummond *et al.*, 2010). Ambiguous nucleotides were represented by IUPAC symbols. Sequences of each DNA region were separately aligned using MAFFT 6 (Katoh *et al.*, 2002) with default parameters, and further adjustments were made by visual inspection. The three cpDNA regions were concatenated in a single matrix.

Standard phylogenetic analyses

Separate phylogenetic analyses were conducted on the ITS and cpDNA matrices using three methods: Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP). Based on previous phylogenetic evidence (Vargas *et al.*, 2004), *Chaenorhinum* was used as the outgroup sequence in all analyses. BI was performed in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) through the CIPRES Science Gateway (Miller *et al.*, 2010). We ran two searches with 10 million generations each and a sample frequency of 1000. Models of nucleotide substitution (Table 2) were selected for each DNA region under the Akaike Information Criterion (AIC) in jModelTest 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). In the cpDNA analysis, DNA regions were partitioned, and substitution models were unlinked across partitions. Fifty-percent majority rule consensus trees with Bayesian posterior probabilities (PP) of clades were calculated after removing the first 10% generations as burn-in. ML analyses were conducted in RaxML (Stamatakis, 2006) under the GTR+G model, with 1000 non-parametric rapid bootstrap replicates. MP analyses were performed in TNT 1.1 (Goloboff *et al.*, 2003), using a "traditional search" with 10,000 replicates saving two most parsimonious trees per replicate, followed by a second heuristic search retaining all best trees and using the trees obtained in the previous 10,000 replicates as the starting ones. The option "collapse trees after the search" was selected in order to discard unsupported nodes. Bootstrap support (MP-BS) of clades was

assessed using 10,000 standard replicates. Significant incongruence between loci prevented us from concatenating the ITS and cpDNA sequences for a total-evidence analysis (Kubatko & Degnan, 2007; Edwards, 2009). Instead, we implemented a species tree estimation analysis (see below).

Dating analyses

Separate ITS and cpDNA matrices including a single individual per species were analyzed through the relaxed molecular clock approach implemented in BEAST 1.6.2 (Drummond *et al.*, 2006; Drummond & Rambaut, 2007). Following previous dating analyses of *Linaria* (Blanco-Pastor *et al.*, 2012), the basal divergence between *Chaenorhinum* and *Linaria* was calibrated using a normal distribution with mean 23 Ma and standard deviation 4 Ma. This was based on a dating analysis of *ndh*F sequences of the tribe Antirrhineae (Appendix 1) which in turn incorporates a calibration of 74 Ma for the divergence time between Oleaceae and Antirrhineae (Bell *et al.*, 2010), and minimum stem-age constraints for Lamiales families and tribes based on five fossils (see Appendix 1 for details). Models of nucleotide substitution (GTR+G for ITS and *trn*K-*mat*K; GTR for *rpl*32-*trn*L^{UAG}; and HKI+I+G for *trn*S-*trn*G) were selected for each DNA region under the AIC criterion in jModelTest. A birth-death process (Gernhard, 2008) was employed as tree prior. The substitution rate variation was modelled using an uncorrelated lognormal distribution. Based on previous estimates for herbaceous plants, uniform prior distributions were set for the substitution rates, with ranges $5x10^{-4}$ to $5x10^{-2}$ substitutions per site per Ma for ITS, and $1x10^{-4}$ to $1x10^{-2}$ substitutions per site per Ma for cpDNA (see Blanco-Pastor *et al.*, 2012 for details). For each dataset, four MCMC analyses with 10 million generations each and a sample frequency of 1000 were run through the CIPRES Science Gateway. Parameter analysis in Tracer 1.5 (Rambaut & Drummond, 2007) showed adequate sample size, with effective sample size (ESS) values above 1000. Chains were combined using LogCombiner 1.6.2, after discarding the first 10% of sampled generations as burn-in. Trees were summarized in a maximum clade credibility (MCC) tree obtained in TreeAnotator 1.6.2 and visualized in FigTree 1.3.1.

Species tree estimation

Phylogenetic incongruence between loci is frequently found in plants, and particularly in *Linaria*, due to incomplete lineage sorting and hybridization, among other causes (Blanco-Pastor *et al.*, 2012). While no standard method is currently available for the inference of phylogenetic relationships in the presence of hybridization, a number of coalescent-based methods have been recently proposed for the inference of species trees that account for incongruence between gene trees caused by incomplete lineage sorting (Liu, 2008; Heled & Drummond, 2010). Here we employed the ITS and cpDNA datasets including one or two individuals per taxon to estimate a species phylogeny of *Linaria* sect. *Versicolores* under the multi-species coalescent method *BEAST (Heled & Drummond, 2010), implemented in BEAST 1.6.2.

Haplotypic data are needed for coalescent-based analyses, which posed a challenge in the case of the multi-copy ITS region. Cloning of ITS copies was not considered due to the low quality of DNA extracts and PCR products obtained from herbarium material. Instead, in order to reconstruct haplotypes from the unphased ITS sequences, we employed the Bayesian statistical method PHASE 2.1 (Stephens *et al.*, 2001; Stephens & Donnelly, 2003), as implemented in DnaSP v5 (Librado & Rozas, 2009), with default parameters (recombination model MR0, 100 iterations, 100 burn-in iterations, thinning interval 1). A Bayesian phylogenetic analysis of the inferred haplotypes was conducted in MrBayes following procedures described above. Given that a close relationship between haplotypes of the same individual was recovered in most cases (and unresolved relationships in some; Supporting Information Fig. S1), the error introduced by potentially incorrect haplotype inference was considered negligible, and all inferred ITS haplotypes were included in subsequent analyses following Blanco-Pastor *et al.* (2012).

Both datasets (ITS and cpDNA) were included as independent loci in the *BEAST analysis. A birth-death process was employed as tree prior. The substitution rate variation was modelled using an uncorrelated lognormal relaxed clock model, with uniform prior distributions for substitution rates as indicated above for dating analyses. Based on results of separate dating analyses of ITS and cpDNA sequences (see above), the crown age of section *Versicolores* was calibrated using a normal prior with mean 6.07 Ma and standard deviation 1.85. Twenty MCMC analyses were run for 100 million generations each, with a sample frequency of 10000. Analysis with Tracer 1.5 confirmed convergence and adequate sample sizes, with ESS values

above 250. Runs were combined using LogCombiner 1.6.2, after discarding the first 10% of sampled generations as burn-in. Trees were summarized in a maximum clade credibility (MCC) species tree obtained in TreeAnotator 1.6.2 and visualized in FigTree 1.3.1. In order to visualize the temporal dynamics of *Linaria* sect. *Versicolores* diversification, lineage-through-time (LTT) plots were generated in the R package *ape* (Paradis *et al.*, 2004) for the MCC species tree and a random sample of 1000 trees from the posterior distribution of the *BEAST analysis.

Analysis of corolla shape

Metric measures

In the personate corolla of *Linaria*, the main reward for pollinators (nectar) is located at the end of an abaxial spur of variable length (Fig. 1). Three main traits determine nectar accessibility to pollinators: spur length, tube width and palate development. Of these, spur length and tube width can be readily measured on herbarium specimens. In order to characterize the inter- and intraspecific variability of these two traits in bifid toadflaxes, both variables were scored for 696 herbarium specimens representing the 30 recognized species and subspecies of *Linaria* sect. *Versicolores* (Supporting Information Appendix S1). Most specimens were provided by the MA, RNG and ATH herbaria. In addition, specimens from the MPU, RAB, FI, K, BM, LD and S herbaria were electronically observed through JSTOR Plant Science (Gallagher, 2010). A single, fully-developed flower per specimen was measured. Spur length was measured from the corolla-calyx insertion to the spur tip (Figs. 2A, 2C). Tube width was measured at the opening level (Figs. 2B, 2D). In addition, the same variables were measured for 377 living specimens collected from 18 Iberian populations of 12 representative species and subspecies sampled for geometric morphometric analyses (see below). All measurements were obtained from scaled digital photographs using ImageJ 1.44p (Abràmoff *et al.*, 2004).

Geometric morphometrics

Geometric morphometrics provides a powerful tool to assess intra- and interspecific variation in flower morphology (Shipunov & Bateman, 2005; Gómez *et al.*, 2006; Abdelaziz *et al.*, 2011). We

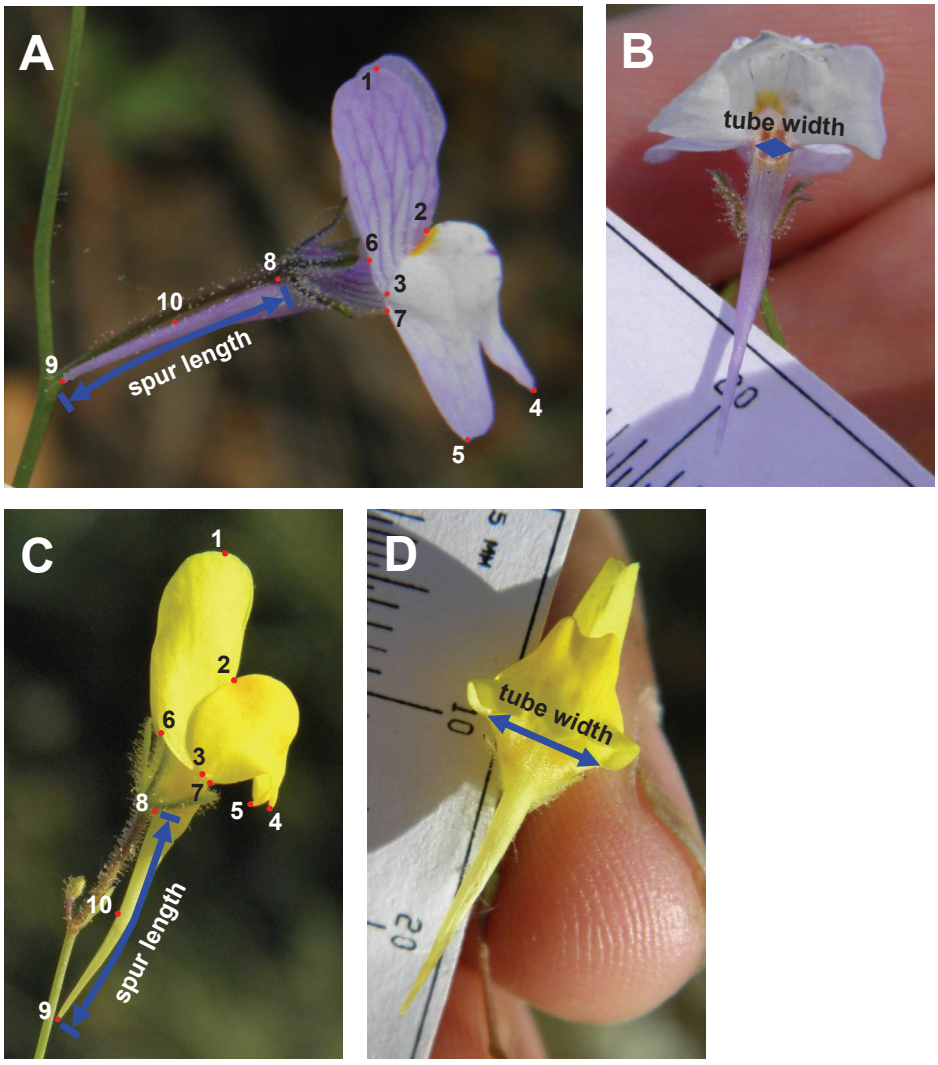


Fig. 2. Metric measures (spur length and tube width) and landmarks (1-10) employed in morphometric analyses, shown in two representative taxa: *L. onubensis* (A, B) and *L. spartea* (C, D). Photos taken in lateral (A, C) and ventral (B, D) view.

used a landmark-based geometric morphometric analysis to describe corolla shape, including palate development, in 18 populations belonging to 12 species and subspecies of *Linaria* sect. *Versicolores* from the Iberian Peninsula (Supporting Information Table S2). These species were considered to represent the full range of corolla shapes of sect. *Versicolores* after our survey of herbarium specimens of all 30 species and subspecies. A total of 369 living specimens (7-25 per population; Supporting Information Table S2) were sampled, and digital photographs were taken of one flower per individual in lateral view (to account for differences in palate development) and planar position. Nine landmarks (Figs. 2A, 2C) were defined at points of evident homology across species (Zelditch *et al.*, 2004): tip of the upper lip lobe (1); union of

upper and lower lips (2); hinge (3); central tip of the lower lip (4); lateral tip of the lower lip (5); intersection of upper lip and corolla tube (6); intersection of lower lip and corolla tube (7); start point of the spur (8); and end point of the spur (9). In addition, one pseudolandmark was defined at the mid point of the spur (10). Landmarks were captured using tpsDig 2.16 (Rohlf, 2010). The two-dimensional coordinates of the landmarks were determined for each individual, and the generalized orthogonal least-squares Procrustes average configuration of landmarks was calculated using the generalized Procrustes analysis (GPA) superimposition method (Rohlf & Slice, 1990; Slice, 2001). Corolla shape differences among species were assessed using a canonical variate analysis (CVA), a multivariate analysis that optimizes between-group differences relative to within-group variation (Albrecht, 1980; Klingenberg & Monteiro, 2005; Abdelaziz *et al.*, 2011). It generates several CV axes and computes between-group Procrustes distances in the CV space. The statistical significance of the between-groups Procustes distances was determined by randomization tests using 10,000 permutations with the software MorphoJ (Klingenberg, 2011).

Evolution of flower morphology

Effect of quantitative traits on diversification rate

We estimated the effect of the two measured quantitative traits (spur length and tube width) on diversification rates of *Linaria* sect. *Versicolores* using the quantitative state speciation and extinction (QuaSSE) method (FitzJohn, 2010). This approach allows the testing of alternative hypotheses of trait-dependent diversification by computing the likelihood of alternative models in which speciation and extinction rates are functions of a quantitative trait that evolves under a diffusion model. We compared the fit of seven models to the evolution of spur length and tube width in bifid toadflaxes: (1) the null (constant) model, with a flat relation between trait values and speciation rate; (2) linear model, with a linear relation between trait values and speciation rate; (3) sigmoid model, with a sigmoidal relation between trait values and speciation rates; (4) hump model, with hump-shaped relation between trait values and speciation rates; (5) linear model with drift; (6) sigmoid model with drift; and (7) hump model with drift. Drift is the directional term of the model, which captures the deterministic component of trait

evolution (FitzJohn, 2010). Models with differential extinction rates were not considered due to the reported low power of the QuaSSE method to detect trait-dependent extinction rates (FitzJohn, 2010). Analyses were implemented in the R package *diversitree* v.0.7-2 (FitzJohn *et al.*, 2009) for the MCC species tree from the *BEAST analysis and 10 additional species trees randomly chosen from the Bayesian posterior distribution, after pruning outgroup taxa. All taxa (species and subspecies) were included in this and subsequent analyses based on the fact that putative intraspecific taxa were usually not closely related in phylogenetic analyses (see below). The maximum-likelihood method was used (Bayesian inference was computationally unmanageable), and model comparisons were performed using likelihood ratio tests.

Effect of overall flower morphology on diversification rate

Morphometric analyses allowed the identification of three major floral morphological types (see Results). We estimated the effect of overall flower morphology on *Linaria* sect. *Versicolores* diversification using the binary-state speciation and extinction (BiSSE) model (Maddison *et al.*, 2007) implemented in *diversitree*. We defined two character states: (0) wide-tubed flower (Types I and II, see below); and (1) narrow-tubed flower (Type III) (Table 1). The fact that Type II was found in a single species (*L. clementei*) prevented us from using the multiple state speciation and extinction (MuSSE) method, which is an extension to BiSSE for more than two character states (e.g. Price *et al.*, 2012). Instead, Type II was grouped with Type I, with whom it is more closely related based on morphometric analyses (see below). The MCC species tree from the *BEAST analysis (with nodes with PP < 0.5 collapsed) and 100 additional species trees randomly chosen from the Bayesian posterior distribution were used to compare state-dependent diversification (speciation and extinction) against nested models with speciation, extinction and transition rate parameters constrained to be equal for both states. We calculated maximum-likelihood parameter values of the unconstrained model (full BiSSE model, 6 parameters) *versus* the constrained models (5 parameters), and the significance of model differences was assessed by performing likelihood ratio tests. We considered that there were no differences in speciation, extinction or transition rates associated to character states when the constrained models were not significantly different from the full model. Parameter values of the full BiSSE model were explored for the MCC tree and a sample of ten additional trees in a

two-step process using maximum likelihood values as a prior for a Markov chain Monte Carlo (MCMC) sampling of parameters, a Bayesian approach (MCMC-BiSSE) which provides a measure of parameter uncertainty (FitzJohn *et al.*, 2009). The ten trees were analyzed with 10,000 steps per tree (chain) and a prior for each parameter exponentially distributed (prioritizing small rates of change, in the absence of evidence to the contrary). After discarding the first 2000 steps of each chain as burn-in, parameter values for each tree were summarized and plotted.

We also estimated net diversification rates for the two major sister groups of sect. *Versicolores* (subsect. *Elegantes* and subsect. *Versicolores*, see below) using Magallón and Sanderson's whole-clade method (Magallón & Sanderson, 2001), as implemented in the R package *geiger* (Harmon *et al.*, 2008). Given that each subsection predominantly displays a different morphological type (Type III for the two species of subsect. *Elegantes* and Type I for 22 out of 28 taxa of subsect. *Versicolores*), comparison of diversification rates was considered useful to further explore the effect of flower morphology on diversification rates. For each subsection, we calculated diversification rates using both crown and stem ages from the *BEAST analysis, and species richness using two different approaches, one synthetic and one analytical, in order to account for uncertainty in species boundaries. In the synthetic approach, we followed the taxonomic treatment shown in Table 1, which includes 23 species of subsect. *Versicolores*. In the analytical approach, subspecies of Table 1 were considered as distinct species, thus making 28 species of subsect. *Versicolores*. The latter approach was based on the fact that intraspecific taxa were usually not closely related in phylogenetic analyses (see below). Following Valente *et al.* (2010b), we calculated diversification rates at two extremes of the relative extinction rate (ε = 0, no extinction; and ε = 0.9, high rate of extinction; where ε = extinction rate/speciation rate), and at the two extremes of the 95% highest posterior density intervals of crown and stem ages.

Finally, we conducted relative cladogenesis tests (Nee *et al.*, 1992; Nee *et al.*, 1994; Purvis *et al.*, 1995) as implemented in *geiger* on a random sample of 100 species trees from the posterior distribution of the *BEAST analysis. This test examines the distribution of numbers of descendents for each branch in the phylogenetic tree existing at some specified time. Under a model with homogeneous rates of cladogenesis, the number of descendents should follow a known distribution. Therefore, lineages with more, or fewer, descendents than expected under

this distribution may be hypothesized as exceptionally successful or unsuccessful (Harmon *et al.*, 2008).

Reconstruction of flower morphology shifts

Ancestral state reconstruction (hereafter ASR) of the two morphological types analyzed in BiSSE was performed in *diversitreee* under the BiSSE model (ASR-BiSSE), thus accounting for differential speciation, extinction and character transition rates in character optimization (Goldberg & Igić, 2008). To account for phylogenetic uncertainty, analyses were separately conducted for the MCC species tree and the same ten additional trees for which parameter values were estimated using MCMC-BiSSE. Parameters distributions obtained in MCMC-BiSSE analyses were used to also account for parameter uncertainty.

For comparison with the maximum-likelihood ASR-BiSSE approach described above, ASR was also performed using parsimony in Mesquite 2.75 (Maddison & Maddison, 2011). In this case, the three morphological types were included, thus treating Type II as a different state than Type I. To account for topological uncertainty, reconstructions were conducted on the full set of species trees of the *BEAST posterior distribution using the "trace character over trees" option. Additionally, the "summarize state changes over trees" option was used to summarize the number of changes between character states across the Bayesian posterior distribution of trees.

Testing of adaptive hypotheses

We tested for the existence of one or more evolutionary optima for spur length and tube width using the maximum-likelihood method implemented in the R package *ouch* (Butler & King, 2004; King & Butler, 2009). Two models based on an Ornstein-Uhlenbeck process (OU) (Hansen, 1997) with one or two optima respectively were tested against a null Brownian motion model. Morphological Types I/II and III were defined as hypothetical selective regimes, and ancestral states were included based on the ASR-BiSSE reconstruction. Model comparisons

were performed using the Akaike information criterion. Analyses were conducted for the MCC species tree and the ten randomly sampled trees employed in QuaSSE and MCMC-BiSSE analyses.

Finally, to analyze the evolution of tube width in relation to spur length in the two main morphological types (Type I and Type III), we used the phylogenetic generalized least squares (PGLS) method (Grafen, 1989), as implemented in the R package *caper* v.0.5 (Orme, 2012). Both variables were log-transformed. Mean values for each taxon were used, and analyses were conducted for the MCC species trees and the same 10 trees used in QuaSSE and MCMC-BiSSE analyses.

Pollinator observations

We performed flower visitor surveys in populations of the twelve Iberian species and subspecies of *Linaria* sect. *Versicolores*, which represent the full range of corolla shapes of the group. A total of 4618 minutes of observations (267 to 941 minutes per taxon) were performed in 2009, 2010, and 2011 at 14 populations that were also included in geometric morphometric and phylogenetic analyses. Visits were considered legitimate when the visitor touched the anthers and stigma. The placement of pollen on the insect body (thorax or proboscis) was recorded.

Results

Phylogenetic relationships and divergence times

The ITS and cpDNA datasets had total aligned lengths of 610 and 2679 bp respectively (see characteristics of the four DNA regions in Table 2). Overall, the BI, ML and MP analyses yielded congruent topologies, except for some weakly supported clades (Fig. 3). The separate phylogenetic analyses of nuclear ITS (Fig. 3A) and cpDNA (Fig. 3B) sequences consistently retrieved section *Versicolores* as a monophyletic group with strong statistical support, and subsections *Elegantes* and *Versicolores* also as monophyletic and sister to each other. Reciprocal monophyly was consistently obtained for the two species of subsect. *Elegantes*. On the contrary,

Table 2. Characteristics of the four DNA regions sequenced for 45 accessions of *Linaria* sect. *Versicolores* and 11 outgroup taxa, and employed in phylogenetic analyses.

	ITS (ITS1-5.8S-ITS2)	*rpl*32-*trn*L^{UAG}	*trn*K-*mat*K	*trn*S-*trn*G
Aligned length (bp)	610	830	1,230	619
Ungapped length range	577-597	582-768	1206-1221	466-590
Pairwise % identity	90.7	94.0	98.2	92.7
Variable characters	199	211	186	139
Parsimony-informative characters	134	106	82	59
Mean % G+C content	56.1	23.6	32.3	27.4
Substitution model	GTR+G	GTR	GTR+G	HKI+I+G

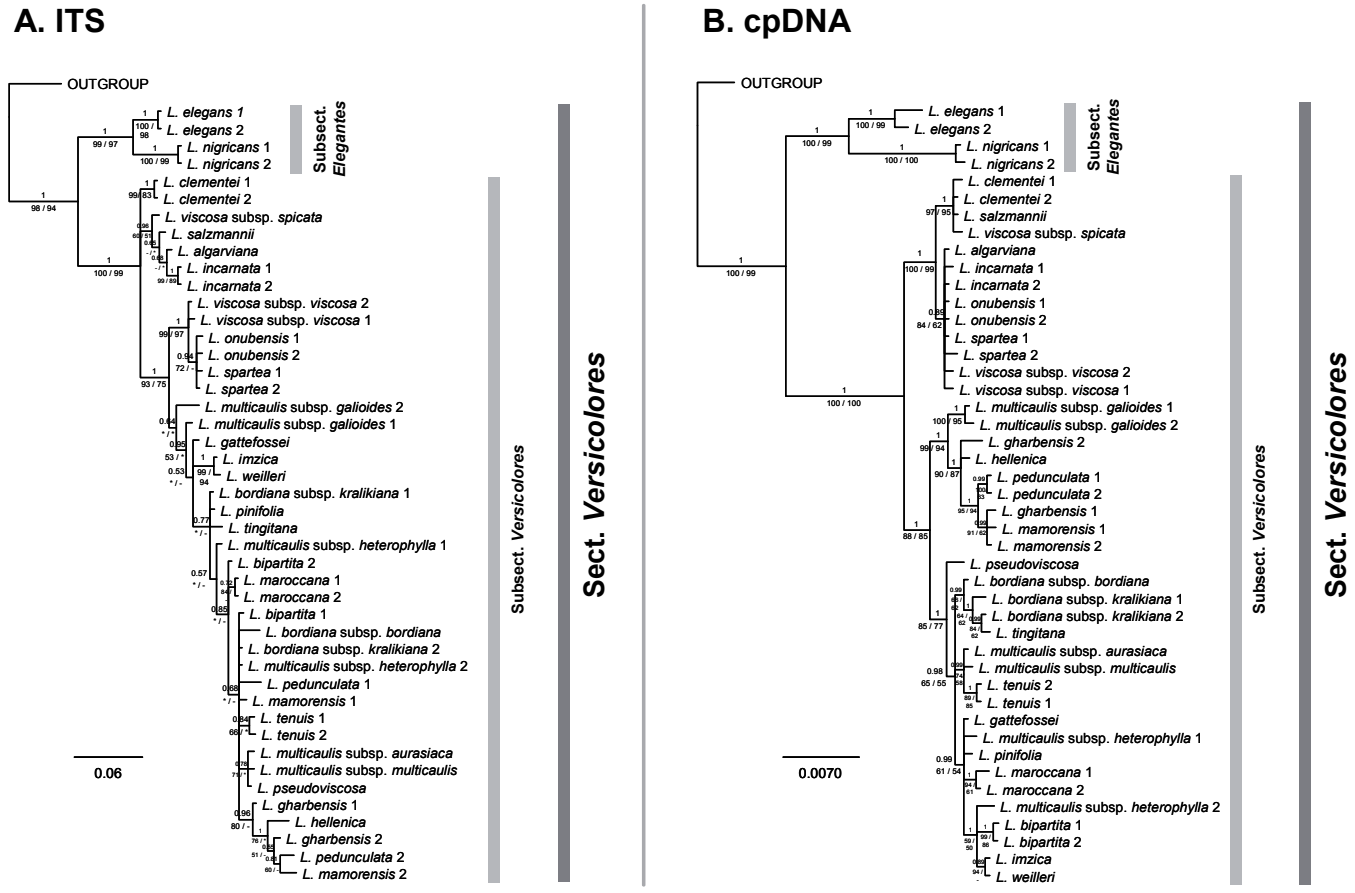


Fig. 3. Phylogenetic analyses of ITS (A) and cpDNA (B) sequences of *Linaria* sect. *Versicolores*. The fifty-percent majority-rule consensus trees obtained in the Bayesian analyses are shown. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood / maximum parsimony percentage bootstrap values. An asterisk (*) indicates no bootstrap support over 50% but clade present in the maximum likelihood tree / strict consensus tree of the maximum parsimony analysis. A hyphen (-) indicates no bootstrap support over 50% and clade absent from the maximum likelihood tree / strict consensus tree of the maximum parsimony analysis. Delimitation of subsections follows Sutton (1988).

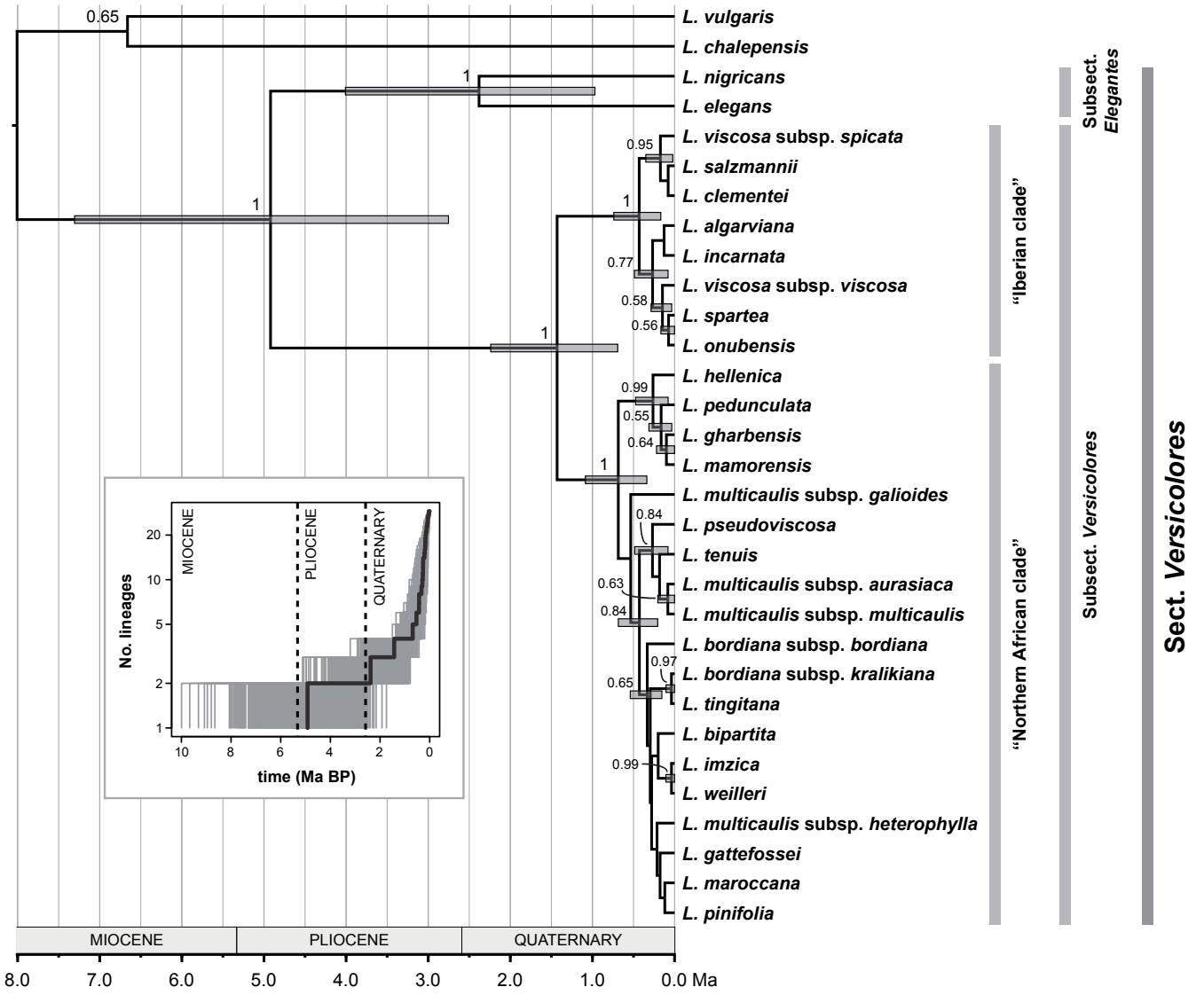
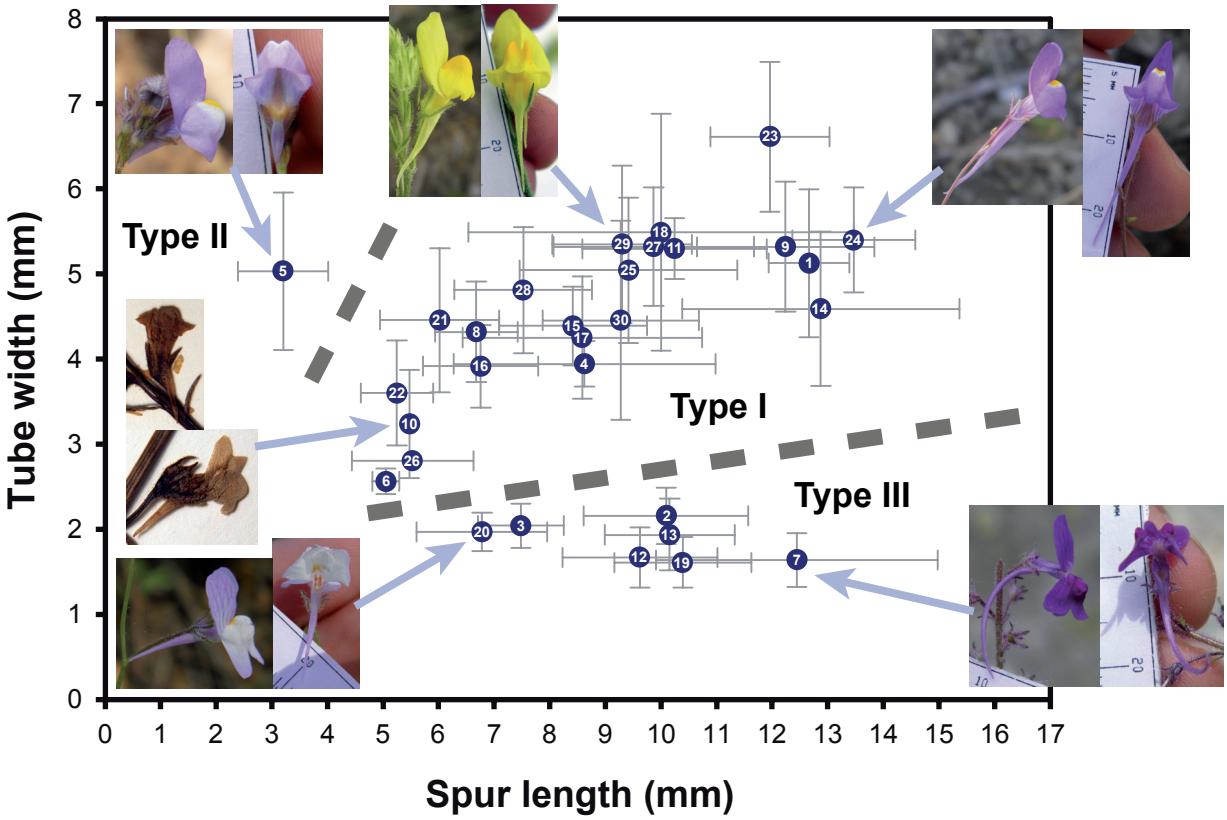


Fig. 4. Time-calibrated species tree obtained in the Bayesian *BEAST analysis based on ITS and cpDNA sequences of *Linaria* sect. *Versicolores*. Node bars represent the 95% highest posterior density intervals for the divergence time estimates. Numbers along the branches are Bayesian posterior probabilities. Major clades are named, including subsections following Sutton (1988). The inset shows a log-lineage-through-time plot for *Linaria* sect. *Versicolores*, based on 1000 trees randomly sampled from the posterior distribution of the *BEAST analysis. The thick line corresponds to the MCC species tree.

topological incongruence between both datasets was extensive within subsect. *Versicolores*. In general, higher clade supports were obtained in cpDNA analyses.

Separate dating analyses based on both loci (Supporting Information Fig. S2) consistently recovered a crown age for section *Versicolores* around 6 Ma BP. This age was then used to calibrate the species tree analysis. The time-calibrated species tree obtained in *BEAST (Fig. 4)



1 *L. algarviana*
2 *L. bipartita*
3 *L. bordiana* subsp. *bordiana*
4 *L. bordiana* subsp. *kralikiana*
5 *L. clementei*
6 *L. dissita*
7 *L. elegans*
8 *L. gattefossei*
9 *L. gharbensis*
10 *L. hellenica*
11 *L. imzica*
12 *L. incarnata*
13 *L. mamorensis*
14 *L. maroccana*
15 *L. multicaulis* subsp. *aurasiaca*
16 *L. multicaulis* subsp. *galioides*
17 *L. multicaulis* subsp. *heterophylla*
18 *L. multicaulis* subsp. *multicaulis*
19 *L. nigricans*
20 *L. onubensis*
21 *L. pedunculata*
22 *L. pinifolia*
23 *L. pseudoviscosa*
24 *L. salzmannii*
25 *L. spartea*
26 *L. tenuis*
27 *L. tingitana*
28 *L. viscosa* subsp. *spicata*
29 *L. viscosa* subsp. *viscosa*
30 *L. weilleri*

Fig. 5. Scatter plot of tube width *versus* spur length measured in 696 herbarium specimens representing the 30 species and subspecies of *Linaria* sect. *Versicolores*. Means (numbered dots) and standard deviations (bars) for each taxon are plotted. The three major morphological types discussed in the text are indicated, and representative taxa are shown.

revealed strongly supported major clades of *Linaria* sect. *Versicolores*, and lower resolution at shallow phylogenetic levels. Divergence between subsections *Elegantes* (PP = 1) and *Versicolores* (PP = 1) was dated back to the late Miocene or Pliocene. Two strongly supported sister clades were recognized within subsect. *Versicolores* (Fig. 4): the "Iberian clade" (PP = 1) included all species that are endemic or subendemic to the Iberian Peninsula, while the "northern African clade" (PP = 1) included all northern African endemics, plus the Ibero-North African

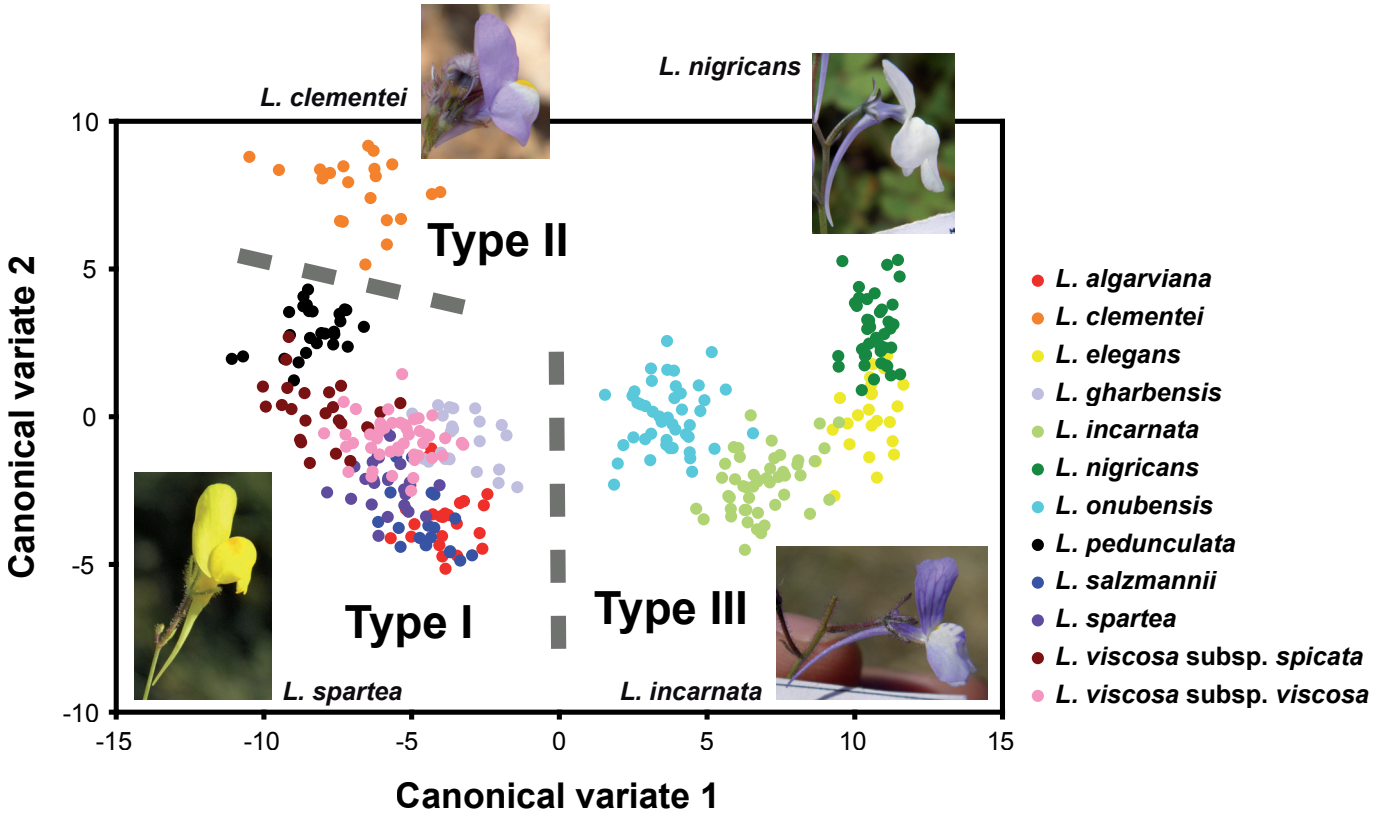


Fig. 6. Canonical variate analysis of the landmark-based geometric morphometric dataset. Scatter plot of canonical variable 2 *versus* canonical variate 1 for 369 living specimens sampled in 18 populations belonging to the 12 Iberian species and subspecies (colours) which represent the full range of corolla shapes of *Linaria* sect. *Versicolores*. The three major morphological types discussed in the text are indicated, and representative taxa are shown.

L. pedunculata and *L. gharbensis*, the sourthern Italian *L. multicaulis* subsp. *multicaulis* and the Greek *L. hellenica*. A Quaternary diversification of subsect. *Versicolores* was estimated, with the Iberian and northern African clades diverging 0.69-2.24 Ma BP, and both clades diversifying in the last million years. The LTT plots (Fig. 4) clearly portrayed a diversification of *Linaria* sect. *Versicolores* mostly in the Quaternary.

Analysis of corolla shape

Measures of spur length and tube width from herbarium specimens of the 30 recognized species and subspecies of sect. *Versicolores* (Fig. 5; Supporting Information Table S3, Fig. S3) revealed three major morphological types. Type I was the most frequent (22 species and subspecies),

and was characterized by a broad tube (> 2 mm) and a wide variation of spur length (4-18 mm). Type II was only found in *L. clementei*, and displayed a broad tube (4-7 mm) and a very short spur (1-4.5 mm). Type III, found in seven taxa, had a narrow tube (1-3 mm) and variable spur length (5-19 mm). The same three groups were consistently recovered when measuring living specimens of the 12 taxa present in the Iberian Peninsula (Supporting Information Fig. S4).

The canonical variate analysis of landmark-based geometric morphometric data of these 12 Iberian taxa (Fig. 6) recovered the same three morphological types. Variation along canonical variate 1 was related to palate development, since it affected the relative position and shape of the upper and lower lips (Supporting Information Fig. S5A; Fig. 6). Variation along canonical variate 2 was related to the shape of the spur and the relative sizes and positions of the upper and lower lips (Supporting Information Fig. S5B; Fig. 6). Taxa of Types I and II displayed well-developed palates, while Type III had broader and spread lower lips. Type II (*L. clementei*) was clearly related to Type I, from which it was mainly differentiated by its short spur.

Evolution of flower morphology

The QuaSSE analyses revealed a significant effect of the two analyzed quantitative traits (spur length and tube width) on speciation rates, as the "constant" model (in which speciation rate does not depend on trait values) was rejected in likelihood-ratio tests for both traits in all analyzed trees (Table 3). The "hump" and "hump with drift" models were recovered as the two best supported QuaSSE models for the two traits. Both models received similar support when analyzing the MCC species tree, as shown by AIC values. Similar results were obtained in the analysis of ten randomly chosen trees (Table 3). Maximum-likelihood parameter estimates for the two models (Table 4) revealed peaks of speciation rate at c. 9 mm for spur length, and c. 4-5 mm for tube width (see xmid in Table 4). For the "hump with drift" model, negative values for the rate of drift were estimated when analyzing the MCC species tree. When analyzing ten randomly chosen trees, all estimates of drift rate were negative for tube width, while they ranged from negative to positive values for spur length.

An effect of flower morphology on diversification rates was supported by the likelihood-ratio tests of BiSSE models (Table 5). In particular, we detected a significant effect on speciation

Table 3. Likelihood-ratio tests of alternative quantitative state speciation and extinction (QuaSSE) models for spur length and tube width. For each trait, six models in which speciation is a constant, linear, sigmoidal or hump-shaped function of the variable are tested against a constant speciation model (speciation rate is independent of the trait). The tests are based on the maximum clade credibility tree of the *BEAST analysis, and the last column additionally summarizes the number of trees (out of a random sample of ten trees from the posterior distribution of the *BEAST analysis) where each model was significantly better (P < 0.05) than the constant model according to a likelihood ratio test. Significance codes: **, 0.001 < P < 0.01; ***, P < 0.001; ns, not significant. Abbreviations and symbols: df, degrees of freedom; lnL, log likelihood; AIC, Akaike information criterion; χ^2, chi-squared statistic; P, P-value.

	df	lnL	AIC	χ^2	P	No. trees with P < 0.05
Spur length						
constant	3	-88.80	183.60			
linear	4	-88.80	185.60	0.00	0.99	0/10
sigmoidal	6	-83.02	178.05	11.55	9.09E-03**	5/10
hump	6	-77.92	167.85	21.75	7.36E-05***	10/10
linear with drift	5	-88.80	187.60	0.00	1.00	0/10
sigmoidal with drift	7	-81.06	176.11	15.49	3.79E-03**	7/10
hump with drift	7	-77.92	169.84	21.75	2.25E-04***	10/10
Tube width						
constant	3	-67.39	140.77			
minimal	4	-67.36	142.72	0.05	0.83	3/10
sigmoidal	6	-66.63	145.25	1.52	0.68	2/10
hump	6	-58.53	129.06	17.71	5.05E-04***	10/10
linear with drift	5	-67.31	144.62	0.15	0.93	3/10
sigmoidal with drift	7	-60.64	135.28	13.50	9.09E-03**	9/10
hump with drift	7	-57.06	128.11	20.66	3.69E-04***	10/10

Table 4. Maximum likelihood parameter estimates of the two best-supported QuaSSE models for trait-dependent (spur length and tube width) speciation. Parameter values for the MCC species tree obtained in the *BEAST analysis are shown. Minimum and maximum values obtained for ten randomly chosen species trees from the Bayesian posterior distribution are shown in square brackets. Parameters: y0, minimum speciation rate; y1, speciation rate at xmid; xmid, trait value with the highest speciation rate; s2, variance of the hump distribution; c, constant; drift, rate of drift; diffusion, rate of diffusion.

	y0	y1	xmid (mm)	s2	c	drift	diffusion
Spur length							
hump	6.35E-06 [7.11E-08–4.97E-02]	12.90 [11.58–46.07]	9.05 [8.70–9.40]	0.80 [0.03–1.08]	5.27 [3.74–7.91]	-	43.19 [20.19–105.73]
hump with drift	5.01E-06 [1.02E-08–1.07E-05]	12.98 [11.63–44.11]	9.07 [8.24–9.54]	0.79 [0.03–1.07]	5.28 [3.84–7.97]	-0.13 [-2.62–2.34]	43.21 [20.48–105.89]
Tube width							
hump	9.77E-06 [2.13E-06–4.96E-04]	9.30 [8.95–26.73]	4.20 [4.03–4.40]	0.25 [0.01–0.22]	4.37 [3.33–6.29]	-	6.85 [4.87–11.70]
hump with drift	1.08E-06 [5.16E-07–8.97E-05]	9.49 [8.92–26.67]	4.86 [4.14-4.97]	0.27 [0.01–0.22]	4.42 [3.33–6.46]	-2.95 [-3.96–-0.40]	5.34 [4.23–10.11]

Table 5. Maximum likelihood estimates of BiSSE parameters and likelihood-ratio tests of alternative models based on the maximum clade credibility tree of the *BEAST analysis. The last column shows the number of trees (out of a random sample of ten trees from the posterior distribution of the *BEAST analysis) where each model was significantly worse ($P < 0.05$) than the unconstrained model according to a likelihood ratio test. Abbreviations and symbols: df, degrees of freedom; λ, speciation rates; μ, extinction rates; q, character transition rates; lnL, log likelihood; AIC, Akaike information criterion; χ^2, chi-squared statistic; P, P-value. Significance code: *, $0.01 < P < 0.05$; ns, not significant.

	df	$\lambda_{I/II}$	λ_{III}	$\mu_{I/II}$	μ_{III}	$q_{I/II \rightarrow III}$	$q_{III \rightarrow I/II}$	lnL	AIC	χ^2	P	No. trees with $P < 0.05$
Unconstrained model	6	3.75	2.83×10^{-6}	3.02	2.33	1.03	7.51×10^{-6}	-23.05	58.10	-	-	-
Symmetric speciation ($\lambda_{I/II} = \lambda_{III}$)	5	2.97	2.97	1.17	6.45	2.05	5.25×10^{-8}	-25.54	61.08	4.97	0.03*	9/10
Symmetric extinction ($\mu_{I/II} = \mu_{III}$)	5	3.53	1.93×10^{-9}	2.61	2.61	1.19	1.55×10^{-7}	-23.09	56.19	0.09	0.77 ns	0/10
Symmetric transition rate ($q_{I/II \rightarrow III} = q_{III \rightarrow I/II}$)	5	4.20	4.45×10^{-9}	3.87	1.77	0.88	0.88	-23.88	57.75	1.65	0.20 ns	0/10

rates, as the "symmetric speciation" model was rejected for the MCC species tree and nine out of ten randomly chosen trees. Bayesian estimation of BiSSE parameters (Fig. 7A-D; Supporting Information Fig. S6) revealed significantly higher speciation rates for morphological Type I/II than for Type III, as shown by the non-overlapping 95% credibility intervals obtained when analyzing the MCC species tree (Fig. 7A) and the ten randomly chosen trees (Supporting Information Fig. S6). No effect on extinction rates was detected (see the widely overlapping 95% credibility intervals in Fig. 7B; Supporting Information Fig. S6). Diversification (speciation

Fig. 7. Analysis of diversification rates and morphological character transitions in *Linaria* sect. *Versicolores*. (A-D) Results of the binary-state speciation and extinction (BiSSE) analysis of the MCC species tree (Fig. 4), considering two character states corresponding to morphological Types I/II and III. Posterior distributions of parameters obtained in the MCMC-BiSSE analysis are shown: speciation rate (A), extinction rate (B), diversification rate (C), and character transition rate (D). Horizontal bars indicate the 95% credibility interval for each parameter. (E) Ancestral state reconstruction of morphological Types I/II and III under state-dependent diversification (ASR-BiSSE), based on parameter estimates shown in A-D. The tree is the MCC species tree with nodes with posterior probability < 0.5 collapsed. The asterisk indicates a clade with a significantly increased diversification rate according to the relative cladogenesis test. (F) Phylogenetic generalized least squares (PGLS) analysis of log(tube width) *versus* log(spur length), separately conducted for morphological Types I and III. Dots represent species and subspecies of Types I (white), II (grey) and III (black). Regression lines and multiple R-squared values for Types I and III are shown.

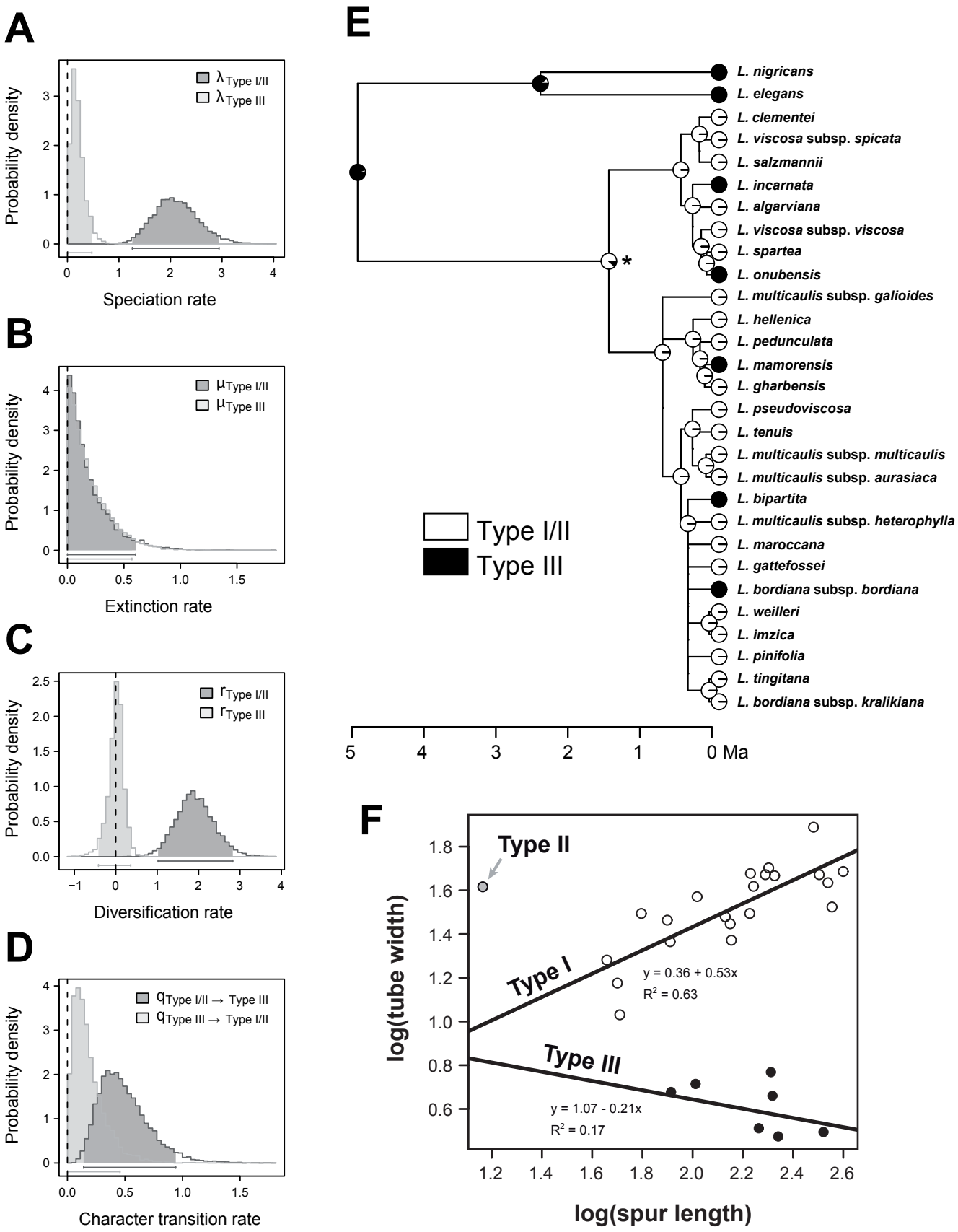

A
Probability density
λType I/II
λType III
Speciation rate
B
μType I/II
μType III
Extinction rate
C
rType I/II
rType III
Diversification rate
D
qType I/II → Type III
qType III → Type I/II
Character transition rate
E
L. nigricans
L. elegans
L. clementei
L. viscosa subsp. spicata
L. salzmannii
L. incarnata
L. algarviana
L. viscosa subsp. viscosa
L. spartea
L. onubensis
L. multicaulis subsp. galioides
L. hellenica
L. pedunculata
L. mamorensis
L. gharbensis
L. pseudoviscosa
L. tenuis
L. multicaulis subsp. multicaulis
L. multicaulis subsp. aurasiaca
L. bipartita
L. multicaulis subsp. heterophylla
L. maroccana
L. gattefossei
L. bordiana subsp. bordiana
L. weilleri
L. imzica
L. pinifolia
L. tingitana
L. bordiana subsp. kralikiana
Type I/II
Type III
5 4 3 2 1 0 Ma
F
Type II
Type I
y = 0.36 + 0.53x
R2 = 0.63
Type III
y = 1.07 - 0.21x
R2 = 0.17
log(tube width)
log(spur length)

Table 6. Net diversification rates of subsections *Versicolores* and *Elegantes*. Estimates based on mean crown and stem ages obtained in the *BEAST analysis are shown, with intervals based on the extremes of the 95% highest posterior density intervals shown in brackets. ε, extinction rate as a fraction of speciation rate.

		Diversification rate (crown age, spp. Ma^{-1})		Diversification rate (stem age, spp. Ma^{-1})	
		ε = 0	**ε = 0.9**	**ε = 0**	**ε = 0.9**
Subsect. *Versicolores*	Synthetic taxonomy (23 species)	1.71 (1.09-3.52)	0.78 (0.50-1.60)	0.64 (0.43-1.14)	0.24 (0.16-0.42)
	Analytical taxonomy (28 species)	1.84 (1.18-3.81)	0.88 (0.56-1.81)	0.68 (0.46-1.21)	0.27 (0.18-0.48)
Subsect. *Elegantes* (2 species)		0 (0-0)	0 (0-0)	0.14 (0.09-0.25)	0.02 (0.01-0.03)

minus extinction) rates were higher for Type I/II (Fig. 7C; Supporting Information Fig. S6), although with certain overlap of the 95% credibility intervals for three out of ten trees. No significant difference between transition rates was found (Fig. 7D; Supporting Information Fig. S6).

Net diversification rate of subsect. *Versicolores* was estimated to be higher than that of subsect. *Elegantes* (Table 6). This result was robust to different extinction rate assumptions, taxonomical approaches and consideration of crown or stem ages. Relative cladogenesis tests recovered consistent results, with the subsect. *Versicolores* clade revealed as significantly more diversified when analyzing the MCC species tree (Fig. 7E) and 89 of 100 randomly chosen trees.

Ancestral state reconstruction under state-dependant diversification (ASR-BiSSE) using the MCC species tree (Fig. 7E) recovered morphological Type III as ancestral to sect. *Versicolores* and subsect. *Elegantes*. Type I/II was inferred as ancestral to subsect. *Versicolores*, which implied an old shift from Type III to Type I/II. Four to five shifts from Type I/II to Type III were inferred within subsect. *Versicolores*, both in the Iberian and northern African clades. Similar results were obtained when conducting ASR-BiSSE analysis on ten randomly chosen trees, although with variable ancestral state probabilities (Supporting Information Fig. S7). Parsimony-based reconstructions yielded congruent results, although with equivocal reconstruction at the root node (Fig. 8A). Accordingly, zero to two shifts from Type III to Type I were estimated when accounting for topological uncertainty (Fig. 8B). Four to six shifts from Type I to Type III were

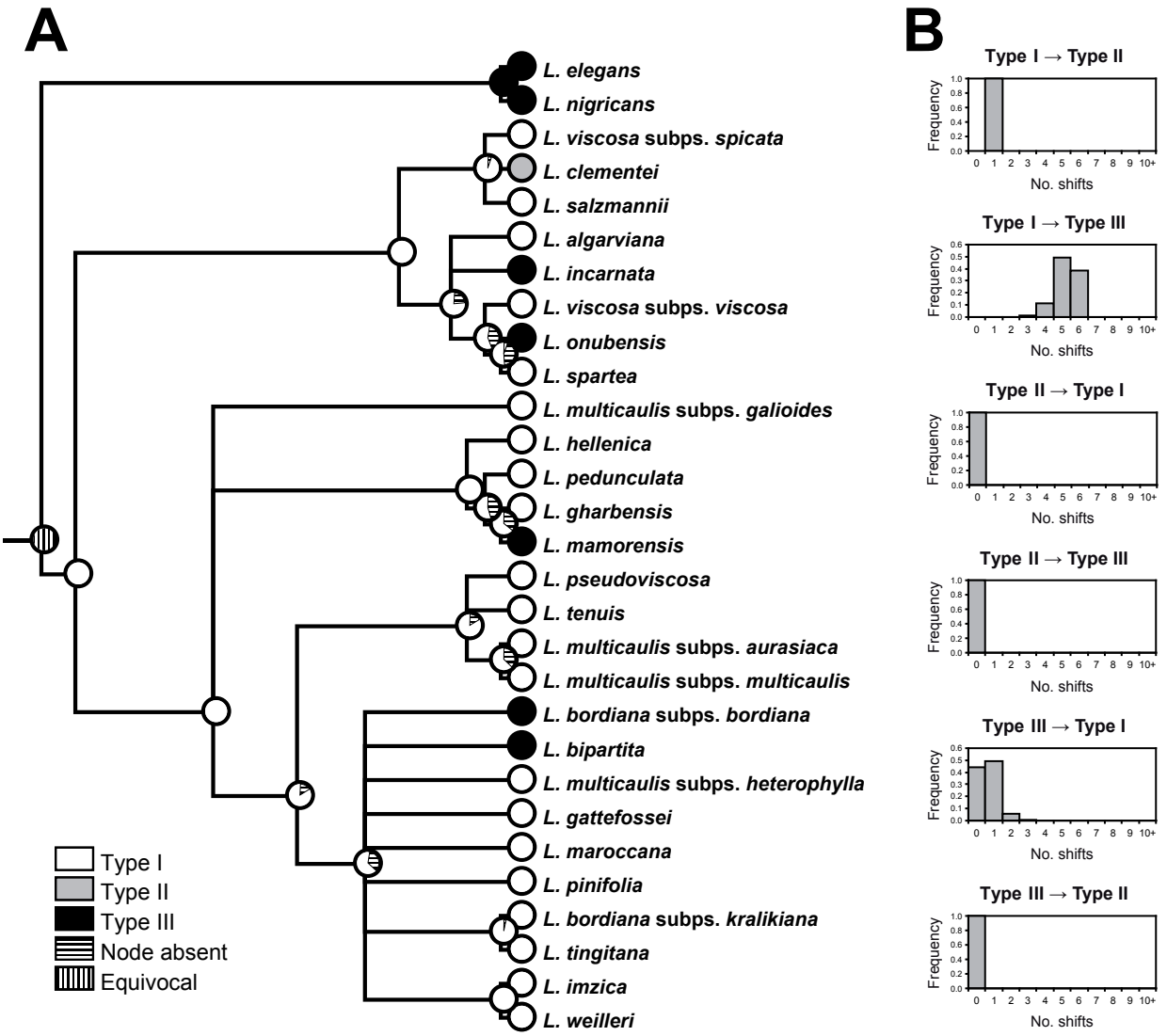


Fig. 8. Parsimony-based ancestral state reconstruction of the three major morphological types of *Linaria* sect. *Versicolores*. (A) Summary of parsimony character optimizations conducted in Mesquite over the full posterior distribution of trees obtained in the *BEAST analysis. The MCC species tree (with nodes with posterior probability < 0.5 collapsed) is shown. Pie charts at nodes summarize the proportion of trees for which a given morphological type was reconstructed at that node. (B) Summary distributions of the number of shifts between character states inferred when implementing parsimony optimization over the full posterior distribution of trees obtained in the *BEAST analysis.

Table 7. Testing of adaptive models. Three models are tested: Brownian motion (BM), Ornstein-Uhlenbeck process with one optimum (OU1), and Ornstein-Uhlenbeck process with two optima (OU2). Values for the MCC species tree obtained in the *BEAST analysis are shown. Optima values for the MCC species tree, and minimum and maximum values obtained for ten randomly chosen species trees from the Bayesian posterior distribution (in square brackets) are indicated. The last column summarizes the number of trees of the same sample where each model was significantly supported based on AICc values. Abbreviations and symbols: *, preferred model; lnL, log likelihood; AICc, corrected Akaike information criterion.

	lnL	AICc	σ^2	α	optima (mm)	No. trees supporting the model
Spur length						
BM	-83.98	172.43	230.45	NA	NA	0/10
OU1*	-68.36	143.68	4891.41	374.84	8.98 [8.94–9.06]	10/10
OU2	-68.10	145.86	2253.02	175.54	8.77 [8.72–8.87], 9.61 [9.57–9.78]	0/10
Tube width						
BM	-62.33	129.12	51.76	NA	NA	0/0
OU1	-51.12	109.20	6301.93	1584.62	3.99 [3.81–4.00]	0/0
OU2*	-32.27	74.20	726.20	669.87	4.67 [4.66–4.68], 1.86 [1.83–1.86]	10/10

estimated, and one shift from Type I to Type II was unequivocally reconstructed. No shifts were obtained from Type II to Types I and III, and from Type III to Type II.

Different adaptive models of trait evolution were supported for spur length and tube width in relation to the two main morphological types (Table 7). When analyzing the MCC species tree, the OU model with one optimum at 8.98 mm was supported for spur length, while the OU model with two optima at 4.67 (Type I/II) and 1.86 mm (Type III) was preferred for tube width. Similar results were obtained for the ten additionally analyzed trees (Table 7). PGLS analyses (Tables 8, 9; Fig. 7F) revealed a positive and significant relationship between tube width and spur length for species of Type I, while no significant relationship was obtained for species of Type III. Similar results were obtained for the MCC species tree and the ten additionally analyzed trees (results not shown).

Table 8. Relationship between tube width and spur length in *Linaria* sect. *Versicolores* species of morphological Types I and III, based on phylogenetic generalized least squares (PGLS) analysis, performed using spur length as independent variable. Multiple r-squared (r^2), phylogenetic signal (**λ**), F-statistics (F) and P-values (P) are shown. Significance code: ***, P < 0.001; ns, not significant.

	r^2	λ	F	P
Type I	0.63	0	$F_{2,19}$ = 32.8	6.89E-07***
Type III	0.17	0.49	$F_{2,5}$ = 1.05	0.42 ns

Table 9. Coefficients of the phylogenetic generalized least squares (PGLS) models for morphological Types I and III.

	Estimate	Std. Error	t value	Pr(> |t|)
Type I				
(Intercept)	0.36	0.20	1.79	0.09
log(spur length)	0.53	0.09	5.73	1.61E-05***
Type III				
(Intercept)	1.07	0.48	2.24	0.07
log(spur length)	-0.21	0.21	-1.02	0.35

Pollinator observations

Observations of flower visitors in the twelve Iberian species and subspecies of *Linaria* sect. *Versicolores* (Table 10) suggested that flowers of Types I and II are mainly pollinated by bees (Hymenoptera) carrying pollen on the back of the thorax, although sporadic visits by nectar-feeding butterflies (Lepidoptera) and bee flies (Bombyliidae, Diptera) carrying pollen on the proboscis were also recorded for three species, including the Type II *L. clementei*. For the four Type III species, a wide variety of flower visitors were observed, most of them sharing a long proboscis: hawk moths (Sphingidae, Lepidoptera), butterflies (Lepidoptera), large anthophorid bees (genera *Anthophora* and *Eucera*; Anthophorini, Apidae, Hymenoptera) and bee flies (Bombyliidae, Diptera). All of them carried pollen on the proboscis.

Table 10. Potential pollinators of Iberian species of *Linaria* sect. *Versicolores*, recorded after 4618 minutes of observations in 2009, 2010 and 2011. Observed strategies of pollen placement on insect body are coded as follows: (T) pollen placed on the back of the thorax; (P) pollen placed on the proboscis; (S) small bees with variable pollen placement; (C) pollen collector. +, >50% of flower visits. -, <5% of flower visits.

Taxon	**Pollinators**
Subsect. *Versicolores*	
L. algarviana	Hymenoptera: *Ceratina saundersi* Daly 1983 (C) + Coleoptera: *Attagenus* sp. (C)
L. clementei	Hymenoptera: *Amegilla quadrifasciata* (de Villers 1789) (T) *Rhodanthidium sticticum* (Fabricius 1787) (T) *Bombus ruderatus* (Fabricius 1775) (T) *Xylocopa* sp. (T) *Heliophila bimaculata* (Panzer, 1798) (T) - *Ceratina mocsaryi* Friese 1896 (S) - Diptera: *Bombylius* sp. (P) Lepidoptera (P)
L. gharbensis	Hymenoptera: *Anthophora plumipes* (Pallas 1772) (T) +
L. incarnata	Diptera: *Bombylius* sp. (P) *Amictus variegatus* (Meigen 1835) (P) Lepidoptera: *Thymelicus lineola* (Ochsenheimer 1808) (P) *Thymelicus sylvestris* (Poda 1761) (P) Hymenoptera: *Lasioglossum* sp. (S) *Ceratina* sp. (S) -
L. onubensis	Hymenoptera: *Eucera nigrilabris* Lepeletier 1841 (P) +
L. pedunculata	Not seen (autogamous species)
L. salzmannii	Hymenoptera: *Heliophila bimaculata* (Panzer, 1798) (T) + *Rhodanthidium sticticum* (Fabricius 1787) (T) *Lasioglossum* sp. (S) *Hoplitis* sp. (S) -
L. spartea	Hymenoptera: *Apis mellifera* Linnaeus 1758 (T) *Heliophila bimaculata* (Panzer, 1798) (T) *Ceratina cucurbitina* (Rossi 1792) (S) - *Lasioglossum* sp. (S) - Lepidoptera: *Euchloe crameri* Butler 1869 (P) -
L. viscosa subsp. *viscosa*	Hymenoptera: *Apis mellifera* Linnaeus 1758 (T) + *Hoplitis* sp. (T) *Xylocopa uclesiensis* Perez 1901 (T) - *Heliophila bimaculata* (Panzer, 1798) (T) - *Lasioglossum* sp. (S) - Lepidoptera: *Euchloe crameri* Butler 1869 (P) -
L. viscosa subsp. *spicata*	Hymenoptera: *Rhodanthidium sticticum* (Fabricius 1787) (T) + *Osmia andrenoides* Spinola, 1808 (T)
Subsect. Elegantes	
L. elegans	Lepidoptera: *Macroglossum stellatarum* (Linnaeus 1758) (P) + *Lasiommata megera* (Linnaeus 1767) (P) - Hymenoptera: *Anthophora retusa* (Linnaeus 1758) (P) - Diptera: *Bombylius major* Linnaeus 1758 (P) -
L. nigricans	Hymenoptera: *Eucera nigrilabris* Lepeletier 1841 (P) + *Apis mellifera* Linnaeus 1758 (P) Lepidoptera: *Colias croceus* (Fourcroy 1785) (P) - *Pontia daplidice* (Linnaeus 1758) (P) -

Discussion

Our phylogenetic analyses based on nuclear and plastid sequences confirmed the monophyly of *Linaria* sect. *Versicolores* (Chapter 2; Appendix 2), and provided strong support for several intra-sectional clades (Fig. 4). Diversification of *Versicolores* has been confirmed to be mostly Quaternary (i.e. in the last 2.6 Ma), as previously suggested based on plastid markers alone (Chapter 3). Fine-scale relationships between species were, however, unresolved or poorly supported in many cases. Low phylogenetic resolution is a common feature of recent radiations (e.g. Hughes & Eastwood, 2006; Scherson *et al.*, 2008; Valente *et al.*, 2010b). It is well known that rapid diversification brings about processes, such as hybridization and incomplete lineage sorting, that may obscure relationships at shallow phylogenetic levels (Degnan & Rosenberg, 2009), as has recently been demonstrated for other lineages of *Linaria* (Blanco-Pastor *et al.*, 2012). Nevertheless, major patterns, such as the sister-group relationship between subsections *Elegantes* and *Versicolores* and the lack of relationship between species of Type III (Fig. 4; Fig. 7), are well supported by our species tree analysis that accounts for incomplete lineage sorting (Heled & Drummond, 2010). Although hybridization cannot be discarded as a source of incongruence between gene trees (Fig. 3), all phylogenetic comparative methods currently available are tree-based (Nunn, 2011). Incorporation of putative hybridization events would have produced reticulated phylogenies that would not have been analyzable for our purposes. Methods based on the multi-species coalescent (Liu, 2008; Heled & Drummond, 2010), rather than concatenated analyses, currently constitute the best approach for the inference of species phylogenies in the presence of incongruent gene trees (Edwards, 2009; Leaché & Rannala, 2011). Incorporation of hybridization to such methods will strengthen phylogenetic inference (Yu *et al.*, 2011; Yu *et al.*, 2012), and the development of comparative methods capable to deal with reticulate phylogenies will probably lead to the reconstruction of more realistic evolutionary scenarios in the future. At present, however, the assumption of a tree-like phylogeny must be made in order to test evolutionary hypotheses using available tools. To account for uncertainty on phylogenetic relationships at shallow levels (part of which might be due to hybridization), we performed all comparative analyses on the consensus species tree and additional samples of likely trees, which had varying sizes depending on the computational demands of each method. Congruence of results across such samples was interpreted as evidence for strong evolutionary signal in spite of phylogenetic uncertainty (Huelsenbeck *et al.*, 2000).

Reversal and convergence in the evolution of flower shape

Although there have been reported other biotic interactions affecting *Linaria* flowers, such as floral herbivory and nectar robbery (Arnold, 1982; Stout *et al.*, 2000; Newman & Thomson, 2005a, b; Sánchez-Lafuente, 2007), insect pollination is likely the most relevant factor affecting the evolution of morphological traits studied here (see Sánchez-Lafuente, 2007). The diverse visitors of *Linaria* sect. *Versicolores* (Table 10) contrasts with the strong large-bee specialization found in larger personate flowers, such as those of *Antirrhinum* (Torres *et al.*, 2001; Vargas *et al.*, 2010), *L. vulgaris* (Stout *et al.*, 2000; Newman & Thomson, 2005a) and several species of *Linaria* sect. *Supinae* (Sánchez-Lafuente, 2007; Sánchez-Lafuente *et al.*, 2011; J.L. Blanco-Pastor, unpublished results). Pollinator diversity was expected at least for two species of sect. *Versicolores* (*L. elegans* and *L. nigricans*) with evident open (not strictly personate) corollas (see Fig. 1P, Q). In addition, the relatively small size of *Versicolores* personate corollas (Sutton, 1988) seems to facilitate their opening by a wide range of pollinators, not only large bees, but also lighter and frailer insects, such as lepidopterans and some dipterans, which are less skilled, in principle, for opening the personate corolla (Table 10). Nevertheless, the presence of pollinator-restrictive traits, such as narrow tubes and long spurs, maintains a specialist pollination system in sect. *Versicolores*, in opposition to another genus of Antirrhineae (*Pseudomisopates*) in which the presence of small, spurless, not fully occluded personate corollas has led to a semi-generalist pollination system (Amat *et al.*, 2011).

Phylogenetic comparative analyses presented here suggest that the evolution of flower morphology in bifid toadflaxes has been dominated by shifts between two morphological types mainly differentiated by the width of the tube and the development of the palate (Figs. 5-8). It is suggested (Table 7) that these two types constitute divergent strategies of pollen placement on nectar-feeding insects (Armbruster *et al.*, 1994; Grant, 1994; Kay, 2006; Yang *et al.*, 2007). These two strategies of *Linaria* pollination (Fig. 9) were first identified by Robertson (1888) and Hill (1909). One strategy corresponds to the typically nototribic pollination of broad-tubed species (Type I/II), in which pollen is deposited on the back of the thorax (scutum) of the nectar-feeding insect (Fig. 9A). This is the strategy found in most *Linaria* species of other sections, and has been demonstrated to result in effective pollination (Macior, 1967; Arnold, 1982; Stout *et al.*,

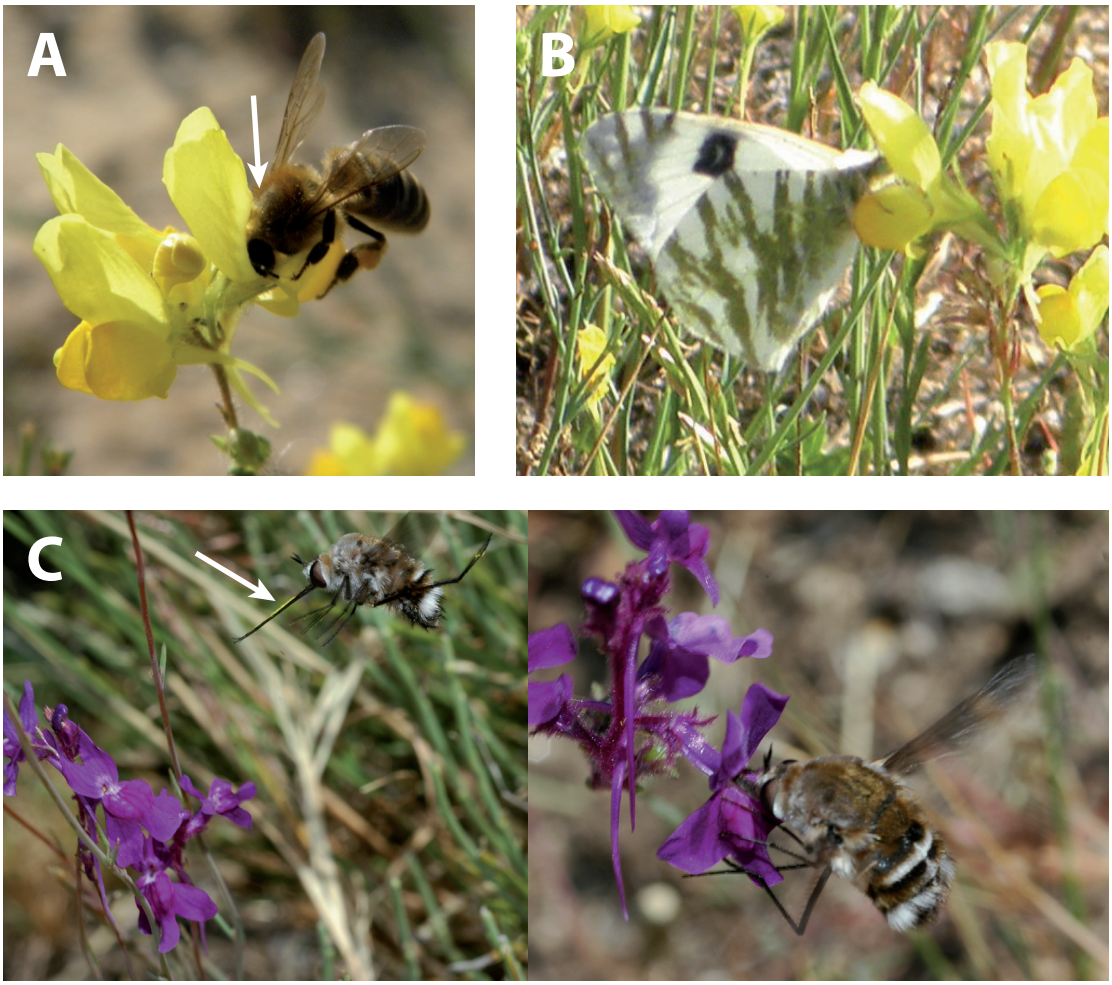


Fig. 9. Different behaviours of potential pollinators of morphological Types I and III. (A) *Apis mellifera* (Hymenoptera) in *L. viscosa* subsp. *viscosa*. (B) *Euchloe* sp. (Lepidoptera) in *L. viscosa* subsp. *viscosa*. (C) Bombyliidae (Diptera) in *L. elegans*.

2000; Newman & Thomson, 2005a; Sánchez-Lafuente, 2007; Sánchez-Lafuente *et al.*, 2011). In section *Versicolores*, the placement of the first optimum inferred by the two-peak OU model (c. 4.67 mm; Table 7) suggests an adaptive adjustment to the thorax width of frequent pollinators. Indeed, closely related species pollinated by similar insects should be similarly selected for the floral phenotype that most efficiently uses these pollinators (Kay & Sargent, 2009). In our case, an adjustment of flower tube size to pollinator size probably maximizes pollen transfer in personate, wide-tubed corollas (but see Vargas *et al.*, 2010). The other strategy is displayed by narrow-tubed species (Type III), in which nectar-feeding insects usually carry pollen on the proboscis (Fig. 9C). In this situation, pollen transfer is maximized by narrowing the tube, so that contact of the proboscis with the anthers and the stigma is guaranteed when the insect

reaches for nectar contained in the spur (Robertson, 1888; Kampny, 1995). This would lead to the second optimum of tube width (c. 1.86 mm; Table 7), which is large enough to fit the anthers (c. 0.5 mm), but narrow enough to guarantee pollen transfer by long-proboscis visitors. The fact that tube width and spur length evolve in a correlated fashion in broad-tubed species (Fig. 7F), but not in narrow-tubed species (1-3 mm width regardless spur length), further suggests that tube width is evolutionary constrained in the latter as an adaptation to long-proboscis pollinators. Similar strategies of pollen placement on pollinators are frequently found in angiosperm lineages with spurred flowers, particularly those pollinated by long-proboscis insects (Herrera, 1993; Johnson & Steiner, 1997; Schiestl & Schlüter, 2009). In *Linaria*, the narrow-tubed strategy has been previously related to pollination by butterflies (Robertson, 1888; Hill, 1909) and moths (Sutton, 1988; Kampny, 1995). Our observations partially support those predictions (see *L. elegans* and *L. incarnata* in Table 10). Additionally, we have found the narrow-tube morphology to be also suited for other long-proboscis pollinators, such as long-tongued bees (mainly from tribe Anthophorini) and bee flies (Bombyliidae) (Table 10).

Several independent origins of similarly configured narrow-tubed flowers have been inferred by ancestral state reconstructions (Fig. 7; Fig. 8). Therefore, this is definitely a case of phenotypic homoplasy, i.e. convergence *sensu* Scotland (2011). In addition, reversal to an ancestral state (which can be regarded as a special case of convergence; Scotland, 2011) is suggested by the ancestral narrow-tubed flower inferred by the ASR-BiSSE analysis, together with its loss and subsequent repeated reappearance in subsect. *Versicolores* (Fig. 7E) (Hall, 2003; Porter & Crandall, 2003). The convergent evolution of narrow-tubed phenotypes in several different lineages of bifid toadflaxes may involve similar genetic and developmental mechanisms (Hall, 2012), in which case it would be interpreted as an instance of parallel evolution *sensu* Scotland (2011) (note that the definitions of convergence and parallelism are controversial, see also Hall, 2003; Hall, 2007; Arendt & Reznick, 2008; Scotland, 2010; Wake *et al.*, 2011; Hall, 2012). Homoplasy of morphological traits can result from common adaptive responses to similar selection pressures, together with genetic and developmental constraints (Wake, 1991; Brakefield, 2006; Wake *et al.*, 2011). While no information about the latter is available for the study group, pollinator observations suggest an adaptive meaning of the two morphological types, as discussed above. Indeed, the repeated shifts from broad-tubed flowers to narrow-tubed ones in subsect. *Versicolores* are not surprising given the fact that

broad-tubed species are visited at a low rate by long-proboscis insects, including butterflies and even bee flies, which carry pollen on their proboscis (Table 10; Fig. 9B). Following the 'pollinator shift' hypothesis (Grant & Grant, 1965; Stebbins, 1970; Campbell, 2008), when a broad-tubed species is faced with an environment in which such long-proboscis insects are dominant, natural selection would favour a narrowing of the tube that would maximize pollen transfer, a mechanism similar to that previously invoked to explain spur elongation in American columbines (Whittall & Hodges, 2007). Conversely, narrow-tubed species can be pollinated by long-tongued bees, particularly those of tribe Anthophorini, which can carry pollen either on the proboscis or on the thorax (Table 10). Selection would then favour tube broadening in the case that pollen transfer by thorax would be more effective than by proboscis. Additional research on reproductive and pollination biology at the population level will be needed to shed further light on the microevolutionary mechanisms (including selective pressures) involved in these putatively adaptive morphological shifts.

Trait dependent diversification

Even though we have inferred a higher number of shifts from broad- to narrow-tubed flowers (4-6) than in the opposite direction (0-1) (Figs. 7, 8), a directional trend in the evolution of morphological types is not supported by BiSSE analyses (Fig. 7D; Supporting Information Fig. S6). Similarly, QuaSSE analyses did not confirm a directional trend for tube width evolution (Table 3). Instead, our analyses of trait-dependent diversification revealed a dissimilar evolutionary success of the broad- and narrow-tube strategies, as shown by the consistently higher speciation rate of broad-tubed species found in BiSSE analyses (Fig. 7A; Supporting Information Fig. S6), together with the speciation peak obtained at 4-5 mm of tube width in QuaSSE analyses (Tables 3, 4). Thus, despite having repeatedly evolved, the narrow-tubed strategy displays limited success in terms of speciation. In fact, a shift from the ancestral narrow tube (as inferred by the ASR-BiSSE analysis; Fig. 7E) to broad tube at the common ancestor of subsect. *Versicolores* may have triggered the Quaternary radiation of this clade (23-28 species), as indicated by relative cladogenesis tests (Fig. 7E). This is also illustrated by the fact that the sister subsect. *Elegantes*, which maintained the ancestral state (narrow tube), displayed a much lower speciation rate, yielding two species during the same period of time (Fig. 4; Table 6). Therefore, our results

indicate that trait-dependent diversification rates, rather than asymmetric rates of change, are responsible for the contrasting species diversities of the two morphological types. The differential diversification success of morphological types is further supported by the fact that additional *Linaria* clades displaying narrow tubes are remarkably species-poor despite being placed at basal phylogenetic positions (sect. *Macrocentrum*, 2 spp.; sect. *Lectoplectron*, 4 spp.) (Chapter 2; Appendix 2).

Previous analyses of evolutionary patterns associated to nectar spurs have focused either on their potential role as key innovations promoting diversification (Hodges, 1997; Hagen & Kadereit, 2003; Ree, 2005; Cacho *et al.*, 2010) or on directional trends in spur length evolution (Whittall & Hodges, 2007). This is the first study in which both trait-dependant diversification rates and directional trends of spur length evolution are jointly analyzed, an approach that is essential in order to obtain sound conclusions about both aspects (FitzJohn, 2010). Our QuaSSE analyses (Tables 3, 4) did not detect a directional trend towards increasingly long nectar spurs, as that described for American columbines (Whittall & Hodges, 2007). In fact, the short-spurred *L. clementei* has recently originated from a long-spurred ancestor (Fig. 8A, B). Transitions to shorter spurs have been demonstrated in other groups (e.g. Micheneau *et al.*, 2008), and may be favoured when the dominant pollinators have tongues shorter than the spur (see Bloch & Erhardt, 2008). On the other hand, we did detect a significant effect of spur length on speciation rates, similar to that found for tube width (Table 3). The detected maximum speciation rate at c. 9 mm was similar to the single adaptive optimum of the best-fit adaptive model (Tables 4, 7). Spur length values centred around 9 mm may be adequate for most of the range of tongue lengths found in nectar-feeding insect pollinators of the Mediterranean region, which may have led to the higher diversification around this value. The lower diversification for longer spurs, and the lack of species with very long ones (i.e. > 20 mm) can probably be related to constrains affecting the two mechanisms that have been hypothesized to account for the evolution of long flower spurs and tubes (reviewed in Johnson & Anderson, 2010): first, the scarcity of pollinators with a very long proboscis may have prevented 'pollinator shifts' associated to further spur elongation (Whittall & Hodges, 2007); and second, the absence of specific, inter-dependent, plant-pollinator mutualistic relationships prevents 'coevolutionary races' of spur and proboscis length (Pauw *et al.*, 2009).

Several flower traits have been previously found to influence diversification rates, including flower symmetry (Sargent, 2004), biotic/abiotic pollination mode (Dodd *et al.*, 1999), and presence of nectar spurs (Hodges & Arnold, 1995; Hodges, 1997). In general, it has been proposed that floral specialization promotes diversification (but see Smith *et al.*, 2008), although it has in turn been suggested that high species diversity may promote floral specialization (Armbruster & Muchhala, 2009). In any case, the relationships between diversification and fine-scale variations of flower traits determining specialization have not been assessed to date in a phylogenetic framework. Here we have shown that traits restricting pollinator access to rewards and pollen placement on pollinators have significant effects on diversification rates of *Linaria* sect. *Versicolores*. Mechanisms of species selection (Stanley, 1975; Jablonski, 2008; FitzJohn, 2010; Rabosky & McCune, 2010) causing such differences in trait-dependent 'emergent fitness' (i.e. heritable differences in net diversification rates) are different to those involved in selection at the individual level (Rabosky & McCune, 2010). Indeed, the narrow-tube strategy in bifid toadflaxes may have recurrently evolved by means of individual-level selection mechanisms, yet it has exerted a negative influence on diversification rates, thus leading to the low frequency of this character state (Fig. 7). Specific mechanisms of species selection acting in bifid toadflaxes may include differential opportunities for exploitation of pollinator fauna (e.g. higher diversity of pollinators carrying pollen on the thorax than on the proboscis) and differential extinction risks (e.g. due to the likely higher specialization of narrow-tubed species). Even though our analyses have detected significant effects on speciation rather than extinction rates, the latter cannot be ruled out given the reported limitations of phylogeny-based methods to detect variation in extinction rates (FitzJohn, 2010; Rabosky, 2010). Anyhow, further research will be needed to understand the relative importance of these and other mechanisms to account for the effects of pollinator-restrictive traits on diversification rates of *Linaria* and other genera with highly specialized personate corollas.

Acknowledgements

We thank Emilio Cano and Fátima Durán for laboratory assistance; Alberto Bañón, Javier Freijanes, Alberto Fernández-Mazuecos, Enrique Sánchez-Gullón, Joaquín Ramírez and Juan Carlos Moreno for field assistance; Concepción Ornosa, Roger Vila and Javier Ortiz for

assistance in pollinator identification; J.J. Aldasoro, B. Estébanez, F. Gómiz, S. Martín-Bravo, E. Rico, the ATH, UPOS and SALA herbaria, and particularly the RNG and MA herbaria, their curators S.L. Jury and M. Velayos, and the *Flora iberica* project for plant material; the "Marismas del Odiel" natural reserve for collection permissions; and J. Quiles, J. Ramírez, E. Rico and O. Fragman-Sapir for permission to use their brilliant photographs. This research was supported by the Spanish Ministry of Science and Innovation through project CGL2009-10031, and by the Spanish Ministry of Education through a FPU fellowship (AP2007-01841) to the first author.

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Supporting Information

Appendix S1. Herbarium specimens studied for taxonomic delimitation and measurement of flower traits (spur length and tube width). Herbarium abbreviations: ATH, Goulandris Natural History Museum, Athens, Greece; BM, The Natural History Museum, London, UK; FI, Natural History Museum, Firenze, Italy; K, Royal Botanic Gardens, Kew, UK; LD, The Natural History Museum, Lund, Sweden; MA, Real Jardín Botánico, Madrid, Spain; MPU, Université Montpellier 2, Montpellier, France; RAB, Institut Scientifique, Rabat, Morocco; RNG, University of Reading, Reading, UK; S, Swedish Museum of Natural History, Stockholm, Sweden.

L. algarviana

Portugal: Cabo de São Vicente, 1-IV-2010, B. Estébanez (MA); Cabo de São Vicente, 24-III-2009, M. Fernández-Mazuecos 11MF09 (MA); Cabo de São Vicente, 14-II-1941, Pinto da Silva (MA).

L. bipartita

Morocco: Bosque de las dunas de Buanamara, V-1913, Dantin (MA); Sidi Bou Knadel, IV-1933, Gattefossé (MA); Mogador, date unknown, Broussonet (MA); Safi, 18-IV-1924, E. Jahandiez (MA); entre Berrechid y Sidi Mohammed el Kebir, 18-IV-1989, M.A. Carrasco *et al.* (MA); Prov. Kenitra, c. 40 km N of Kenitra on road to Moulay Bousselham, 25-V-2002, S.L. Jury 19270 (MA); Safi, pr. oppidulum Tamri, loco dicto Assif Tamri, 23-V-1985, C. Blanché *et al.* (MA); Safi, pr. oppidulum Tamri, loco dicto Assif Tamri, 23-V-1985, C. Blanché *et al.* (MA); dunas de Buanamara, camino a Larache, date unknown, Dautin (MA); Rabat, by gate to botanic garden of Institut Agronomique et Vétérinaire Hassan II at entrance to club ACSA, 22-I-2000, S.L. Jury 18558 (RNG); Larache, entre Ksar-el-Kebir y Larache, cerca de la fábrica de azúcar, 17-III-1995, M.A. Mateos *et al.* (RNG); 57 km NW of Agadir, main road to Essaouira 4 km N of Tamri, 18-III-1994, S.L. Jury 14293 (RNG); c. 50 km S Larache, rd Souk El Arbaa to Moulay-Bousselham, 24-II-1994, S.L. Jury 13217 (RNG); Safi, pr. oppidulum Tamri, loco dicto Assif Tamri, 23-V-1985, C. Blanché *et al.* (RNG); Hab. in arenosis, c. El Araix, l. El Mensah, 8-II-1930, Font Quer (RNG); between Tamri and Cap Rhir, 1-IV-1972, Davis 53945 (RNG); Cap Beddouza (N of Safi), 7-IV-1972, Davis 54169 (RNG); between Oualidia and Cap Beddouza, 25-III-1972, D. Bramwell *et al.* (RNG); above Sidi Mousa (nr. Tiznit), 19-III-1972, Davis 53551 (RNG); 5 km S of Cap Beddouza, Sidi Bouchta, 29-III-1974, Miller, Russell & Sutton 195 (RNG); 50 km NE of Marrakech, near Tamelelt, Miller, Russell & Sutton 792 (RNG); 25 SW of Tafraoute, between Tiznit and Tafraoute, 3-IV-1974, Miller, Russell & Sutton 651 (RNG); Essaouira, wooded sand-dunes along coast S of Essaouira, 26-III-1972, D. Bramwell, I.B.K. Richardson & B.G. Murray 196 (RNG); route de Rabat à Témara, 13-IV-1976, J. Lewalle 8309 (RNG); 1 km S of El-Jadida, El-Jorf-Lasfar, 29-III-1974, Miller, Russell & Sutton (RNG); 2 km S of Rabat, El Harhoura, 28-III-1974, Sutton, Miller & Russell 91 (RNG).

L. bordiana* subsp. *bordiana

Algeria: Bou Tlélis, forêt de M'Sila, 25-III-1932, R. Le Cesve (RNG); 20 km E of Oran, Kristel to Aïn Franin, 16-IV-1976 D.A. & S.J. Sutton 248 (RNG); Bou Tlélis, forêt de M'Sila, 25-III-1932, R. Le Cesve (MA); El Ancor, alrededores de Oran, IV-1922, Ch. d'Alleizette (MA).

L. bordiana* subsp. *kralikiana

Algeria: 55 km E of Mostagenem, E of Sidi Lakhdar, 14-IV-1976, D.A. & S.J. Sutton 172 (RNG); 49 miles from Mostaganem to Tenes, 30-IV-1971, Davis 51833 (RNG).

L. clementei

Spain: Málaga, Alhaurín de la Torre, 5-V-2008, M. Fernández-Mazuecos 7MF08 (MA); Málaga, puerto de Ojén, entre Ojén y Coín, 15-VI-1994, A. Aparicio *et al.* 7404 MV (MA); Málaga, cerro de Carratraca, Sierra Blanquilla, 12-V-1979, P. Cantó *et al.* 1065 GF (MA); Málaga, pr. Carratraca, 25-V-2008, C. Aedo 15732 (MA); 6 km südlich Coin, zwischen Coin und Mijas, 21-V-1999, M. Nydegger 36574 (MA); Málaga, Mijas, 29-VIII-1980, G. López 2473 GF (MA); Málaga, Yunquera, 24-V-1999, C. Aedo *et al.* CN-2295 (MA).

L. dissita

Algeria: O. Hauts plateaux à Arbaout, VI-1857, A.N. Pomel (MPU); Hauts plateaux Oranais, Mou el Gtouta, V-1862, A.N. Pomel (MPU).

L. elegans

Portugal: Tras-os-Montes, Vila Real, Montalegre, Meixedo, 10-VI-2010, A. Herrero *et al.* AH4209 (MA); Tras-os-Montes, Vila Real, Montalegre, Pitoes das Junias, 9-VI-2010, A. Herrero *et al.* AH3993 (MA).

Spain: Cuenca, El Tobar, 5-VI-2009, M. Fernández-Mazuecos 55MF09 (MA); Cáceres, Eljas, Puerto de San Martín, 15-VI-2009, M. Fernández-Mazuecos 58MF09 (MA); Ávila, Puerto de Peña Negra, 8-V-2009, M. Fernández-Mazuecos & P. Vargas 41MF09 (MA); Cuenca, Santa María del Val, 5-VI-2009, M. Fernández-Mazuecos 56MF09 (MA); Pontevedra, Caldas de Reis, 12-V-2009, M. Fernández-Mazuecos 43MF09 (MA); Madrid, Somosierra, 3-VII-2008, M. Fernández-Mazuecos & A. Bañón, 32MF08 (MA); Ávila, Puerto del Tremedal, 5-VII-2008, M. Fernández-Mazuecos 33MF08 (MA); León, Villafrea de la Reina, 10-VII-2008, M. Fernández-Mazuecos & D. Orgaz 39MF08 (MA); León, Isoba, subiendo hacia el puerto de San Isidro, 11-VII-2008, M. Fernández-Mazuecos & D. Orgaz 40MF08 (MA); Ávila, Puerto de Casillas, 27-VI-2008, M. Fernández-Mazuecos & B. Estébanez 27-VI-2008 (MA); Burgos, Neila, carretera hacia Villavelayo, 26-VI-2009, M. Fernández-Mazuecos & B. Estébanez 72MF09 (MA); Zamora, Ribadelago, entre Ribadelago Nuevo y Ribadelago Viejo, 29-VII-2008, M. Fernández-Mazuecos 48MF08 (MA); Orense, San Xoán de Río, 29-VII-2008, M. Fernández-Mazuecos 48MF08 (MA); Ávila, Navarredonda de Gredos, frente al Parador, 22-VI-2008, M. Fernández-Mazuecos 29MF08 (MA); Burgos, subiendo hacia el pico Cerezales, 26-VI-2009, M. Fernández-Mazuecos 73MF09 (MA); Salamanca, Navasfrías, Puerto Viejo, 15-VI-2009, M. Fernández-Mazuecos 57MF09 (MA); La Coruña, Santiago de Compostela, junto a la vía del tren, 12-V-2009, M. Fernández-Mazuecos 42MF09 (MA); Madrid, Puerto de Canencia, 26-VI-2008, M. Fernández-Mazuecos (MA); Ávila, Puerto de Menga, 22-VI-2008, M. Fernández-Mazuecos 28MF08 (MA); Ávila, Puerto del Pico, hacia el Risco del Duque, 22-VI-2008, M. Fernández-Mazuecos 27MF08 (MA); La Coruña, Brión, Pedrouzos, 21-V-2006, J. Amigo (MA); Lugo, Sierra de Ancares, cumbre del Mustallar, 28-VI-1982, S. Castroviejo *et al.* 6963 SC (MA); La Rioja, Mansilla de la Sierra, río Cambrones, 28-V-1988, J. Arizaleta *et al.* 3045 JP (MA); Teruel, Sierra de Albarracín, date unknown, Almagro (MA); Teruel, Fuente del Puerto, Orihuela a Bronchales, 13-VI-1988, G. Mateo (MA); La Rioja, Mancomunidad de Canales de la Sierra, Mansilla y Villavelayos, barranco Cambrones, 30-V-1993, M.L. Gil Zúñiga & J.A. Alejandre (MA); Cáceres, Puerto de Tornavacas, 2-V-1987, E. Villanueva & I. Benítez 722EVG (MA); Burgos, roquedos sobre el Arlanzón, 19-VI-1976, E. Fuentes (MA); Ávila, Puerto de Mijares, montaña Gamonosa, 5-VII-2008, A. Quintanar *et al.* AQ2917 (MA); Cuenca, Beteta, fuente de Pérez, 13-V-2010, Ó. García Cardo, OGC-01308 (MA); Madrid, El Escorial, Puerto de la Cruz Verde, 10-VII-2008, P. Vargas *et al.* 134PV08 (MA); a la derecha del camino de los prados de S. Bartolomé, El Tobar, Cuenca, 12-VI-1942, A. Rodríguez (MA).

L. gattefossei

Morocco: Beni-Mellal, al E de Tizi-N-Aït-Ouirra, 1-VI-1998, F. Gómiz (MA); província de Beni Mellal, región de El Ksiba, 5-VII-2006, A. Quintanar *et al.* AQ2025 (MA); Quercetum sur terrain décalcifié, au Tizi n'Aït Ouirah, El Ksiba, 28-VI-1929, J. Gattefossé (RAB).

L. gharbensis

Morocco: Temara, 24-I-1986, J. Lewalle 11270 (MA); Meknès-Tafilalet, Azrou vers El-Hajeb, 2-IV-2010, T. Buira *et al.* JC4472 (MA); Rabat, Avicenne, 15-II-1993, J. Lewalle 13880 (MA); Tiflet, 14-IV-1996, J. Lewalle 14027 (MA); Tetuán, 21-V-1928, Font i Quer (MA); Hefrán?, 6-IV-1926, Vidal y López 533 (MA); NE of Chefchaouen, near mosque W of Bou Ahmed, 26-II-1994, S.L. Jury 13406 (RNG); Rabat, by gate to botanic garden of Institut Agronomique et Vétérinaire Hassan II at entrance du club ACSA near Soukaina Mosque, 22-I-2000, S.L. Jury 18559 (RNG); Chefchaouen, Bab Taza, cumber de Bab el Karn, sobre el collado de la pista a Fifi, 19-VI-1993, J. Monstserrat & J. Vicens JMM-4193/5 (RNG); Tetouan, Souk el Arba de Beni Hassim, Tayenza, 6-VI-1996, M.A. Mateos & J.M. Montserrat JMM-5453/4 (RNG); 16 km S of Rabat, Temara –plage, 28-III-1974, Sutton, Miller & Russell 120 (RNG); Temara, 24-I-1986, J. Lewalle 11270 (RNG); Cabo Negro, 15 km N of Tetouan, 10-IV-1971, Davis 51160 (RNG); Tetuán, 21-V-1928, Font i Quer (RNG); 40 km S of Rabat, 29-III-1974, Miller, Russell & Sutton 156 (RNG).

Spain: Huelva, Gibraleón, junto a Vía Verde, 17-III-2009, M. Fernández-Mazuecos *et al.* 7MF09 (MA); Huelva, Gibraleón, Vía Verde del Litoral, 9-IV-2008, E. Sánchez-Gullón (MA); Huelva, Los Medios, Aljaraque, 26-IV-2006, E. Sánchez-Gullón (MA); Huelva, Cartaya, 3-IV-1996, E. Sánchez-Gullón (MA).

L. hellenica

Greece: Peloponnisos, prov. Lakonia, distr. Epidhavros/Limiras, settlement of Kambos, Neapolis (Vion), 27-III-1959, collector unknown (ATH).

L. imzica

Morocco: ladera NE del Jbel Imzi, Antiatlas, 6-IV-2004, F. Gómiz (MA); Sous-Massa-Daraâ, Anezi, Agadir-ogjgal, jbel Imzi, ladera norte, 6-VI-2009, J. Calvo *et al.* JC3813 (MA); Antiatlas occidental, ladera NE Jbel Imzi, 13-IV-2007, T. Buira & J. Calvo 232 (MA).

L. incarnata

Portugal: Alto Alentejo, Montemor-o-Novo, Escoural, estrada entre Escoural e Casa Branca, 9-III-2006, D. Espírito Santo *et al.* (MA); Pinhel, entre Ervas Tenras e Malta ao km 109.7, 28-VI-1970, Regeira, Serra do Bernardino (MA); Alto Alentejo, Serra de S. Mamede, prox. da Torre Caldeira, 5-V-1957, Malato-Beliz *et al.* (MA); Casa-Branca, IV-1915, G. Sampaio (MA); Beira Litoral, Coimbra, pinhal de Marrocos, 26-V-1954, A. Matos & A. Marques (MA); Beira Baixa, Penamaçor, Serra da Malcata, 12-V-1970, J. Malato-Beliz & J.A. Guerra (MA); Alto Alentejo, entre Santa Eulalia e Monforte, 2-IV-1954, Malato-Beliz (MA); Alto Alentejo, Nisa, Nossa Senhora da Graça, 4-V-1971, Malato-Beliz & J.A. Guerra (MA).

Spain: Cáceres, Valencia de Alcántara, near ermita de Barbón, 27-IV-1994, Optima Inter VI 884 (RNG); Cáceres, borde de carretera en término de Torrejón el Rubio, 9-VI-1993, J.L. Pérez Chiscano (RNG); Mozárbez (Espagne, prov. Salamanca), route vers Monterrubio de la Sierra, 15-V-1983, M. Ladero & F.J. González (RNG); Badajoz, Alburquerque, finca El Hito, 24-III-2009, M. Fernández-Mazuecos 9MF09 (MA); Zamora, cerca de Bercianos de Aliste, los Carballos, 31-V-10, B. Estébanez & N. García (MA); Salamanca, Pelabravo, arroyo Gargabate, 5-V-2009, M. Fernández-Mazuecos & P. Vargas 39MF09 (MA); Cáceres, Valencia de Alcántara, Majadallana, 8-IV-2004, M. de la Estrella 3 (MA); Cáceres, Valencia de Alcántara, cercanías de la ermita de Barbón, 27-IV-1994, VI Itinera Mediterranea 884 (MA); Morille, Salamanca, 16-V-1998, E. de Paz (MA); Monfragüe, pantano del Tiétar, Cáceres, 21-IV-1987, A. Segura Zubizarreta 34555 (MA); Cáceres, Torrejón el Rubio, 24-III-1982, Ladero *et al.* (MA); Cáceres, borde de carretera en término de Torrejón el Rubio, 9-VI-1993, J.L. Pérez Chiscano (MA); suelo arenoso-lignoso plio-cuaternario de km 43 de carretera de Torrejón el Rubio a Trujillo, Cáceres, 12-IV-1977, J.L. Pérez Chiscano (MA); Cáceres, Torrejón el Rubio, Monfragüe, 10-IV-1980, D. Belmonte (MA); Badajoz, Alburquerque, 19-IV-1978, J.L. Pérez Chiscano (MA); suelo sobre granitos del término de Santibáñez Alto, Cáceres, 16-IV-1991, J.L. Pérez Chiscano (MA).

L. mamorensis

Morocco: ctra. Kenitra-Khemisset, a unos 20 kms de Kenitra, 17-IV-2006, S. Martín-Bravo *et al.* 34SMB06 (MA); Tánger-Tétouan, Larache, pr. Lixus, 9-IV-2009, J. Calvo & I. Espejo JC3481 (MA); 12 km S of Marrakech, road to Asni, 19 km N of Tahanoute, 15-III-1994, S.L. Jury 14151 (MA); abords de la forêt de la Mamora, près de l'aéroport de Rabat-Salé, 15-III-1977, M. Atbib (MA); Kenitra, IV-1933, J. Gattefossé (MA); c. El Araix, l. El Mensah, 8-II-1930, Font Quer (MA); reg. Rabat, 10 km W de Tiflete, 1955, Sauvage (MA); prov. de Salé 7 km a l'E de Salé, route vers Meknès, Layayda, 18-III-1995, J. Lambinon 95/Ma/333 (MA); playa de Tánger, IV-1921, C. Pau (MA); Larache, 1914, Pérez Camarero (MA); Mamora, Aïn Jorra, 1-V-1924, E. Jahandiez (MA); prov. de Salé 7 km a l'E de Salé, route vers Meknès, Layayda, 18-III-1995, J. Lambinon 95/Ma/333 (RNG); Rabat, forêt de la Mamora, carretera de Rabat a Meknès, km 17, 24-V-1994, M.J. Díez *et al.* (RNG); Tetouan, entre Asilah y Larache, Tnine-Sidi-El-Yaman, 20-IV-1988, Silvestre *et al.* (RNG); 12 km S of Marrakech, road to Asni, 19 km N of Tahanoute, 15-III-1994, S.L. Jury 14151 (RNG); Safi, prope Tamri, 1-V-1992, Fernández-Casas 13710 & Molero (RNG); sol sableux, siliceux, près de la formet de la Mamora près Aéroport-Rabat-Salé, 15-III-1977, M. Atbib (RNG); near Sidi Yahya du Rhab, E of Rabat, 2-III-1982, I.R. Smith 33 (RNG); Forêt de la Mamora au NE de Rabat, 4-I-1980, F. & J. Damblon 80/5 (RNG); diplomatic forest between Tangier and Asilah, 6-IV-1971, Davis 51003 (RNG).

L. maroccana

Morocco: a 4 km de Boujad, cerca de Kasba-Tadla, 12-VI-1982, Fernández-Casas *et al.* FC 6738 (MA); Marrakech, inter Tahnaout et Oukaïmedene, prope oppidulum Tadmamt, 28-V-1985, C. Blanché *et al.* (MA); Tlata Ida Gougmar, carretera S-7076, 5-III-2001, J. Arrington *et al.* (MA); Agadir, Col du Kerdouss, 24-V-1985, C. Blanché *et al.* (MA); High Atlas, S of Marrakech, on road to Tizi-n-Test, near Mosque at Tin Mal, 16-III-1994, S.L. Jury 14209 (RNG); High Atlas, Imouzzer Valley, N of Agadir, 2006, M. Ait Lafkih (RNG); Marrakech, inter Tahnaout et Oukaïmedene, prope oppidulum Tadmamt, 28-V-1985, C. Blanché *et al.* (RNG); High Atlas, Asni, 7 km from Tahanaoute on road to Oukaimeden, 13-VI-1974, Reading Univ. / B.M. Exped. 675 (RNG).

L. multicaulis* subsp. *aurasiaca

Algeria: c. Aurès, Ras Pharaoun, 18-VI-1874, A.N. Pomel (MPU).
Tunisia: below El Kesra, East of Maktar, 3-V-1975, Davis & Lamond D.57154 (RNG).

L. multicaulis* subsp. *galioides

Morocco: High Atlas, c. 70 km from Marrakech, c. 400 m below Oukaïmeden along road to Marrakech, 23-IX-2000, S.L. Jury 18628 (RNG); High Atlas Vallée du Zat, Yagour, 'Zib Zguigui, 2-VI-2007, A. Kool 904 (RNG); High Atlas, S of Marrakech, ski resort of Oukaïmeden, 25-VII-1997, S.L. Jury 18089 (RNG); Oukaïmeden village and environs, 17-VII-1989, M. Ait Lafkih *et al.* 525 (RNG); 72 km S of Marrakech, Oukaïmeden, 3-VII-1987, S.L. Jury *et al.* 8844 (RNG); prov. de Marrakech, Hoher Atlas, an der Straße nach Oukaimeden, ca. 6 km unterhalb Oukaimeden, 13-VII-1989, D. Podlech 48008 (MA); prov. de Marrakech, Hoher Atlas, Umgebung von Oukaimeden und Berge S des Ortes, 14/16-VII-1989, D. Podlech 48067 (MA); High Atlas, S of Marrakech, ski resort of Oukaïmeden, 25-VII-1997, S.L. Jury 18089 (MA); distrito de Beni-Mellal, en la pista de Aït Mhammed a Ifrane, desviación a Zawyat Oulmzi, 4-VI-2006, Medina *et al.* LM3548 (MA); Alto Atlas, Adrar-n-Oukaïmeden, vertiente N, 29-VI-2006, A. Herrero *et al.* AH 3035 (MA); Marrakech, Oukaïmedene, pista por encima de la estación de esquí, 11-VII-1984, G. López & F. Muñoz Garmendia (MA); Alto Atlas, Oukaimeden, alrededores del poblado, 6-VII-1997, J. Güemes *et al.* JGH-1610 (MA); Ouarzazate, El Kelaa des Mgouna, Amesker, Tizi N'Aït Hamad, 17/18-VII-1984, G. López & F. Muñoz Garmendia 9189 GL (MA); Alto Atlas, Jbel Anngour, pr. Oukaimeden, 6-VII-1997, J. Güemes *et al.* JGH-1617 (MA); Alto Atlas, Jbel Siroua, entre Amassine y Tala, poco después de Tizi-n-Tleta (Tizi Touggoukine), 30-VI-1997, J. Güemes *et al.* JGH-1494 (MA); djebel Ghat Demnet, IV-1885, J. Ball (FI); South Marocco, Greater Atlas, Revaia, V-1871, Hooker (K).

L. multicaulis* subsp. *heterophylla

Algeria: 10 miles NE of Mostaganem, 30-IV-1971, Davis 51853 (RNG); Dj. Djurdjura, near Tikjda, 3-VI-1971, Davis 53078 (RNG); Tikdjda, parque nacional Djurdjura, 19-VII-2011, J.J. Aldasoro A-19066 (MA).

Morocco: Meknès, ad lacum Aguelmame Azigza dictum, 3-VI-1985, C. Blanché *et al.* (RNG); Middle Atlas, prov. Ifrane, 5.3 on road from Azrou to Midelt, 6-VI-2007, S.L. Jury & R. Shkwa 20911 (RNG); Marrakech, cerca de Oukaimeden, Tazenah, 2-VI-1980, Fernández-Casas FC 3342 (RNG); Atlas Medio, Jbel bou Drâa, al norte del Jbel Hedri, 13-VI-1996, M.A. Mateos & J.M. Montserrat JMM-5991/4 (RNG); Chefchaouen, Talembote, Jbel Tazaout, 21-VI-1993, J. Montserrat *et al.* JMM-4228/5 (RNG); Taza, Jbel Tazzeka, 11-VI-1996, M.A. Mateos & J.M. Montserrat JMM-5865/2 (RNG); Taza, Djebel Berkane, 25-V-1994, M.J. Díez *et al.* (RNG); Central Rif, Jbel Tighighine, 13-VI-1995, A. Boratynski & A. Romo R-8651/6 (RNG); Targuist, carretera de Targuist a Al Hoceima, Jbal el Bâbet, a 6 km de Beni Hadifa, 12-V-1994; F. Bombardó *et al.* JMM-5253/4 (RNG); High Atlas, Ayachi, NW facing cliffs Ayachi, 20 km from Midelt, 22-VI-1974, Reading Univ. / B.M. Exped. 952 (RNG); carretera Azrou-Midelt, a unos 5 km de Azrou, 16-V-2008, M. Fernández-Mazuecos & J.C. Moreno 16MF08 (MA); Taza, Jbel Tazekka, 23-VI-2008, E. Rico *et al.* SA-221 (MA); carretera Azrou-Midelt, a 7 km de Azrou, 16-V-2008, M. Fernández-Mazuecos & J.C. Moreno 15MF08 (MA); Ifrane, a la izquierda de la carretera hacia Boulemane, 16-V-2008, M. Fernández-Mazuecos & J.C. Moreno 12MF08 (MA); Jbel Hebri, 18-V-2008, M. Fernández-Mazuecos & J.C. Moreno 17MF08 (MA); Tizi-n-Tretten, 16-V-2008, M. Fernández-Mazuecos & J.C. Moreno 13MF08 (MA); Marrakech, cerca de Oukaimeden, Tazenah, 2-VI-1980, Fernández-Casas FC 3342 (MA); hab. in cistetis, supra emporium Sok-et-Tnin d. (Beni Hadifa), 26-V-1927, Font Quer (MA); Middle Atlas, prov. Ifrane, 5.3 km along road from Azrou on way to Midelt, 6-VI-2007, S.L. Jury & R. Shkwa 20911 (MA); Azrou, bosque de cedros, 13-VI-1990, M.A. Carrasco *et al.* (MA); Beni-Hadifa, ctra. hacia Targuist, 23-IV-2004, J. Martínez *et al.* 108bBGA04 (MA); Col de Tanout ou Fillal, 6-VII-2006, A. Quintanar *et al.* AQ2106 (MA); Azrou, pista forestal hacia Aïn Leuh, 23-VII-1984, G. López & F. Muñoz Garmendia (MA); Moyen Atlas, fôret de Bikrit, 4-VII-1996, S. Cirujano *et al.* (MA); Ksar Es Souk, col du Zad, 19-VI-1982, Fernández-Casas *et al.* FC 7111 (MA); Atlas Medio, S de Timhadit, 26-VI-1997, C. Aedo *et al.* CA 4239 (MA); Jbel Lakra, collado encima de la casa forestal, 20-VI-1997, E. Rico *et al.* 6004ER (MA); Atlas Medio, refugio de Taffert, 25-VI-1997, C. Aedo *et al.* CA 4231 (MA).

Tunisia: Tabarka, 3-V-2002, J.J. Aldasoro A-2888 (MA); gobernación de Nabeul, península del Cap Bon, cercanías de Taklisah, 31-III-2009, A. Quintanar *et al.* AQ3264 (MA).

L. multicaulis* subps. *multicaulis

Italy: Sicilia, Catania, cerca de Bronte, 6-VI-2000, Álvarez *et al.* IA 1570 (MA); Sicilia, Palermo, La Pizzuta, pr. Portella della Páglia, 30-V-2000, Aedo *et al.* 5713 (MA); Sicilia, Catania, Parco dell Etna, Bosco Cerrita, 6-VI-2000, Álvarez *et al.* IA 1622 (MA).

L. nigricans

Spain: Almería, Tabernas, 21-IV-2009, M. Fernández-Mazuecos & P. Vargas 31MF09 (MA); Almería, Tabernas, 13-III-2008, P. Vargas 3PV08 (MA); Almería, Cabo de Gata, junto a las Salinas, 24-III-2010, M. Fernández-Mazuecos *et al.* 18MF10 (MA); Almería, San Juan de los Terreros, playa de la entrevista, 13-IV-1993, G. Aragón & I. Martínez GA 0070 (MA); Almería, Tabernas, Los Retamares, 21-III-2012, J. Calvo *et al.* (MA); Almería, Tabernas, 17-III-1984, G. Mateo (MA); Almería, Cuevas de Almanzora, Pozo del Esparto, 27-III-1998, A. Carrillo *et al.* CN 1835 (MA); Almería, Tabernas, 19-III-1984, G. Mateo & R. Lázaro (MA); Almería, Tabernas, cerca de la carretera a Sorbas, 14-III-1999, M.B. Crespo *et al.* (MA); Almería, entre Venta de los Yesos y Tabernas, 20-V-1976, B. Cabezudo *et al.* (MA).

L. onubensis

Spain: Huelva, Fuente de la Corcha, 27-IV-2010, M. Fernández-Mazuecos & A. Bañón 28MF10 (MA); Huelva, entre La Palma del Condado y Valverde del Camino, 20-VI-1978, S. Talavera & B. Valdés (MA); Huelva, Riotinto-Campofrío,

17-IV-1980, J. Rivera *et al.* (MA); entre Trigueros y Valverde, prov. de Huelva, 11-IV-1960, S. Rivas Goday *et al.* (MA); Valverde, Huelva, 13-V-1931, E. Gros (MA).

L. pedunculata

Portugal: Playa de Monte Gordo, 6-V-2010, M. Fernández-Mazuecos & J.L. Blanco-Pastor 61MF10 (MA); sand dunes, Armaceo de Pera, Algarve, 5-IV-1967, R.M. Wadsworth *et al.* (RNG).

Morocco: entre Rabat y Casablanca, Mohammedia, playa de Assanoubar, 16-IV-1984, Aparicio *et al.* (RNG); Chefchaouen, playa de Targha, 8-IV-1995, A.J. Caruz *et al.* (RNG); Tetuan, Oued-Laou, 14-III-1995, M.A. Mateos *et al.* (RNG); 14 km SE of Tetouan, Cap Mazari, 9-IV-1974, Miller, Russell & Sutton 846 (RNG); Restinga, Tetouan-Ceuta, 9-IV-1971, Davis 31102 (RNG); Tetouan, Larache, Ras Remel, 20-IV-1988, Silvestre *et al.* (RNG); Gharb – Chrarda – Béni Hassen, Moulay-Bousselham, 3-IV-2010, T. Buira *et al.* JC4502 (MA); Tánger – Tétouan, Tleta-de-Oued-Laou, 7-IV-2009, J. Calvo & I. Espejo JC3460 (MA); c. 4 km NW of Oued Laou, 26-II-1994, S.L. Jury 13364 (MA); hab. in arenosis maritimis, pr. El Araix, 23-III-1930, Font Quer (MA); hab. in arenosis maritimis l. Rincón de Medik, inter Ceuta et Tetauen, 13-III-1930, Font Quer (MA).

Spain: Huelva, Lepe, urbanización Nueva Umbría, 5-V-1978, B. Cabezudo *et al.* (RNG); prope Gibraltar, Hispania, prov. Cádiz, Baetica, 23-V-1922, E. Gros (RNG); circa Cádiz, Hispania, prov. Cádiz, Baetica, 26-III-1925, E. Gros (RNG); Vejer de la Frontera, Los Caños de Meca, Cádiz, 27-IV-1978, T. Luque *et al.* (RNG); Cádiz, Cádiz Isthmus, 18-IV-1951, A.H.G. Alston (RNG); Cádiz, Zahara de los Atunes, 27-IV-1978, T. Luque *et al.* (RNG); Cádiz, entre Punta Palomas y Tarifa, 16-IV-1974, S. Talavera & B. Valdés (RNG); Almería, Roquetas de Mar, Punta El Sabinar, a 200 m del faro, 11-IV-1997, M.B. Crespo *et al.* (MA); Huelva, Marismas del Odiel, 17-III-2009, M. Fernández-Mazuecos *et al.* 3MF09 (MA); Málaga, Faro Calaburras, Mijas Costa, 17-IV-2009, M. Fernández-Mazuecos & J. Ramírez 27MF09 (MA); Almería, Cabo de Gata, playa de las Salinas, 21-IV-2009, M. Fernández-Mazuecos 30MF09 (MA); Huelva, Islantilla, 9-III-2004, E. Sánchez-Gullón (MA).

United Kingdom: Gibraltar, ladera E del Peñón, entre Catalan Bay y Dudley Ward Way, 17-5-1985, J. Bensusan *et al.* (MA).

L. pinifolia

Algeria: El Kala ex La Calle, bord de la route allant d'El Kala au Cap Rosa, près des rives ouest du Lac Melah, 7-VII-1979, A. Dubuis *et al.* (RNG); 7 km W of El Kala, La Calle, 11-V-1971, Davis 52172 (RNG); El Kala ex La Calle, bord de la route allant d'El Kala au Cap Rosa, près des rives ouest du Lac Melah, 7-VII-1979, A. Dubuis *et al.* (MA); in arenosis ditionis Senhadja prope lacum Anatum, 21-VI-1934, R. Maire (MPU).

L. pseudoviscosa

Tunisia: Kairouan, in camp. arenos., 15-V-1896, S. Murbeck (BM); Kairouan, in campis arenosis, 15-V-1896, S. Murbeck (LD); Kairouan, in campis arenosis, 15-V-1896, S. Murbeck (S); 6 km SW of El Haouaria on road to Soliman, Nabeul province, 2-V-1990, P. Wilkin & E.J. Wellens (RNG).

L. salzmannii

Spain: Málaga, El Chorro, Sierra de Almorchón, 17-IV-2009, M. Fernández-Mazuecos & J. Ramírez 19MF09 (MA).

L. spartea

Portugal: Praia da Adraga, 25-III-2009, M. Fernández-Mazuecos 13MF09 (MA); Montemor-o-Novo, 26-III-2009, M. Fernández-Mazuecos 18MF09 (MA); Alto Alentejo, Mora, a 7 km de Pavia, 18-IV-1987, A. Moura (MA); Sierra da Estrella, VI-1966, M. Gilbert (MA); Baixo Alentejo, Serpa-Pias, 12-IV-1949, F. Fontes & B. Rainha (MA); Alto Alentejo, Serra de Ossa, 1954, Malato-Beliz (MA); Alto Alentejo, Serra de S. Mamede, 12-VI-1959, M. Beliz & J.A. Guerra (MA); Alto Alentejo, Mourao, 21-III-2001, S. Castroviejo *et al.* SN329 (MA); Évora Monte, entre Estremoz y Évora, 28-V-

1996, M.A. Carrasco *et al.* 13663SC (MA); próximo a Vilar Formoso, 17-VII-1983, E. Bayón *et al.* 8741 SC (MA); carretera de Alcobaça a Nazaré, 3 km antes de Nazaré, 23-IV-1978, P. Cubas *et al.* 057MG (MA); Estremadura, Faro, 13-VI-1961, M.-Béliz & J.A. Guerra (MA); Algarve, entre Aljezur e Odesseixe, 27-IV-1956, Malato Beliz *et al.* (MA); Baixo Alentejo, Peninsula de Troia, 17-V-1973, Malato-Beliz & J.A. Guerra (MA).

Spain: Badajoz, Alburquerque, finca El Hito, 24-III-2009, M. Fernández-Mazuecos 10MF09 (MA); Huelva, Marismas del Odiel, La Cascajera, 17-III-2009, M. Fernández-Mazuecos *et al.* 5MF09 (MA); Toledo, Velada, camino hacia la ermita de la Virgen de Gracia, 15-IV-2008, M. Fernández-Mazuecos 3MF08 (MA); Huelva, El Romerano, 1-IV-2008, E. Sánchez-Gullón (MA); Salamanca, entre Calvarrasa de Arriba y Arapiles, 5-V-2009, M. Fernández-Mazuecos & P. Vargas 40MF09 (MA); Zamora, Fonfría, Castro de Alcañices, 31-V-2010, B. Estébanez & N. Medina (MA); León, Villalís de la Valduerna, 2-VI-2010, B. Estébanez & N. Medina (MA); León, Alija del Infantado, 1-VI-2010, B. Estébanez & N. Medina (MA); Madrid, Colmenar Viejo, base del cerro de San Pedro, 24-VII-2007, P. Vargas 101PV07 (MA); Badajoz, Calamonte, non longe minis ab urbe Mérida, 9-II-1982, S. Castroviejo 6158 SC (MA); Cantabria, Páramo de la Lora, Valderredible, 2-IX-1983, E. Loriente (MA); Orense, Cudeiro, en los alrededores de la capital, 15-XI-1987, J. Amigo (MA); Pontevedra, 11-VI-1916, L. Crespi (MA); Castrelo de Miño, Orense, 17-VII-1935, A. Rodríguez (MA); Pontevedra, Tui, Caldelas de Tui, augas abaixo do balneario, 8-V-1996, X.R. García Martínez 6527 (MA); Jaén, Cambil, alto del Mercadillo, 21-V-1985, C. Fernández (MA); J. Andújar, Sierra Quintana, 23-VI-1985, C. Fernández & E. Cano (MA); Jaén, Andújar, pantano del Jádula, 4-V-1983, E. Postigo (MA); Huelva, Sanlúcar de Guadiana, El Romerano, 24-IV-2008, E. Sánchez-Gúllón (MA); Huelva, Sierra de Aracena, entre Valdeflores e Higuera de la Sierra, arroyo del Rey, 24-II-1978, J. Rivera (MA); Córdoba, C-110, entre Villaviciosa y Córdoba, alto de la carretera en el km 15, 6-V-1988, E. Bayón *et al.* (MA); Sevilla, entre Puebla del Río y Venta del Cruce, 7-V-1988, E. Bayón *et al.* (MA); Jaén, Baños de la Encina, 21-V-1992, F. Gómez Manzaneque & C. Morla (MA); Badajoz, Cabeza del Buey, 5-III-2000, P. Escobar García (MA); Cáceres, Losar de la Vera, Valle del Tiétar, Vega del Cincho, 20-III-1980, Meana *et al.* (MA); Badajoz, Campanario, VI-1911, V. Lagares (MA); Cáceres, Talayuela, 8-VI-1975, E. Valdés-Bermejo (MA); Cáceres, orilla del río Salor, pr. Aliseda, 2-V-1988, Aedo CA 4459 (MA); Cáceres, Garganta de la Olla, 3-VI-1993, G. Aragón *et al.* (MA); Badajoz, Santa Amalia, río Gúdalo, 20-III-2001, J.J. Aldasoro 1208 (MA); Cáceres, Valencia de Alcántara, Majadallana, 8-IV-2004, M. De la Estrella 9 (MA); Badajoz, pr. Olivenza, 22-III-2001, C. Aedo *et al.* CA 6062 (MA); Baños de Montemayor, Cáceres, VII-1904, C. Escribano (MA); Quintana de la Serena, Badajoz, 11-V-1971, B. Casaseca (MA); Badajoz, Campanario, IV-1971, Fernández-Casas (MA); Soria, el Royo, a orillas del río Zazón, 16-VIII-1983, J. Molero & A. Rovira (MA); Cueto del Moro, León, 1944, Rojas (MA); León, El Bierzo, prope Ponferrada, 17-V-1933, W. Rothmaler (MA); Gordaliza del Pino, León, 10-VI-1990, P. Montserrat (MA); Burgos, Carazo, camino de la ermita de la Virgen del Sol, 12-VII-1979, Pons-Sorolla & Susanna (MA); Burgos, Covarrubias, Cerezuelos, 24-V-1998, M. Rodrigo Juarros (MA); Burgos, Covarrubias, frente a la granja Degesa, 7-VI-1998, M. Rodrigo Juarros (MA); Zamora, Tábara, Regato del Correo, 25-V-1996, P. Bariego Hernández (MA); Valladolid, 22-VI-1906, F. Sennen (MA); Castellanos de Villiquera, Salamanca, 7-VI-1967, B. Casaseca (MA); carretera de Salamanca, La Alberca, Salamanca, 23-VI-1946, A. Caballero (MA); Ávila, Valle de Amblés, La Torre, 15-V-1976, Fuertes & Laredo (MA); Ávila, Castronuevo, 19-VI-1984, Barrera *et al.* (MA); Ávila, Ramacastañas, 30-IV-1987, Luceño & Vargas PV2025bis (MA); Segovia, Revenga, 11-VI-1988, R. García Adá 5037 RG (MA); Segovia, Pedraza, de Pedraza a Matabuena, 6-VI-1987, R. García Adá (MA); Segovia, Aguilafuente, 18-VI-1988, R. García & G. López 5200 RG (MA); Segovia, La Granja de San Ildefonso, de Pradera de Navalhorno a La Granja, 14-VI-1985, R. García 777 RG (MA); Segovia, Laguna de la Tenca, Lastras de Cuéllar, 10-IX-1989, C.J. Martín CJM 203 (MA); Cuenca, La Mota del Cuervo, desvío a San Clemente, 10-V-1978, G. López 516 GF (MA); Ciudad Real, Villamayor de Calatrava, volcán del Morrón de Villamayor, 14-V-2000, M. Bellet & C. Santamaría (MA); Albacete, Villarrobledo, entre Villarrobledo y El Provencio, 3-V-2008, A. Buira & J. Calvo JC2182 (MA); Guadalajara, La Fuensaviñán, Navazo del Pozo, 24-VI-1982, J. Baranda *et al.* 6266 SC (MA); Valdenuño, prov. Guadalajara, 30-XII-1992, P. Garin (MA); Madrid, Valle del Paular, 10-VI-1977, Gutiérrez Bustillo & M. Costa (MA); Madrid, San Agustín de Guadalix, 28-VI-1982, J.C. Moreno (MA); Madrid, Aldea del Fresno, 8-VI-1984, Carrasco *et al.* (MA); Madrid, Fuente del Fresno, 12-III-1977, M. Pastrana 434SC (MA); Madrid, Torrelaguna, 10-XI-1972, E. Valdés

Bermejo (MA); Madrid, El Pardo, 6-V-1934, J. Cuatrecasas (MA); Madrid, Móstoles, Rancho Grande, 5-II-1958, E. Guinea (MA); Madrid, Dehesa de Arganda, 30-IV-1966, Bellot & Monasterio (MA); Ávila, El Barraco, Las Zauderas, 28-IV-2004, B. García Muñoz & I. Sánchez Tejedor (MA); Cáceres, Malpartida de Plasencia, 5-V-1983, E. Bayón *et al.* (MA).

L. tenuis

Libya: Tripoli, nr. University of Libya, 12-III-1970, Davis 49462 (RNG).

Tunisia: gobernación de Gabès, Matmata, pr. M'Dou, 23-III-2009, C. Aedo *et al.* CA16263 (MA); El-Djem, III-1909, C.J. Pitard (MA).

L. tingitana

Algeria: 55 km E of Mostagenem, E of Sidi Lakhdar, 14-IV-1976, D.A. & S.J. Sutton 173 (RNG); 25 km W of Mostaganem, La Macta, 15-IV-1976, D.A. & S.J. Sutton 208 (RNG); 25 km W of Mostaganem, 2 km SE of El Macta, 18-IV-1976, D.A. & S.J. Sutton 383 (RNG); La Macta, pres Mostaganem, 9-IV-1933, A. Faure (MA); Mazagran, les Sablette, à environ 6 km au SW de Mostaganem, 27-VI-1985, A. Dubuis (MA).

L. viscosa* subsp. *spicata

Spain: Málaga, Canillas de Albaida, 7-V-2008, M. Fernández-Mazuecos *et al.* 10MF08 (MA); Granada, Sierra Nevada, Peñones de San Francisco, 14-VII-2010g, J.L. Blanco-Pastor 147JB10 (MA); Jaén, Villanueva de Arzobispo, carretera hacia El Tranco, 25-V-2009, M. Fernández-Mazuecos 45MF09 (MA); Jaén, Cazorla, Puente de las Herrerías, 25-V-2009, M. Fernández-Mazuecos 49MF09 (MA); Málaga, Canillas del Aceituno, pista hacia Sierra Tejeda, 19-IV-2009, M. Fernández-Mazuecos & J.L. Blanco-Pastor 28MF09 (MA); Málaga, Sierra Tejeda, Barranco Roque, 24-IV-1987, S. Castroviejo 10003SC (MA); Jaén, Villanueva del Arzobispo, Sierra de las Villas, 3-V-1985, C. Soriano (MA); Málaga, Sierra Almijara, Nerja, carril Fuente del Esparto, 23-IV-2009, J. Ramírez (MA); Central de Diechar, 22-VI-1922, collector unknown (MA); Málaga, Sedella, Sierra de Almijara, 14-VI-1994, A. Aparicio *et al.* 7351 MV (MA); Albacete, San Juan de Alcaraz, 5-VII-1891, Porta & Rigo (MA); El Gallinero, pr. Riópar, reg. Murc., 12-VII-1923, Cuatrecasas (MA); Granada, Sierra de Alfacar, Alfaguarilla, 18-VI-1979, Pérez Raya & Molero Mesa (MA); Jaén, Jamilena, 14-V-1982, C. Fernández (MA); Granada, Puerto de la Mora, cra. al Pozuelo, a 2.5 km de la general, 12-VI-1989, G. Nieto-Feliner 2724GN & A. Izuzquiza (MA); laderas arenosas y cunetas de la carretera pr. Pinos Prados, en la Sierra de Cázulas, Granada, 13-V-1990, A. Pallarés (MA); Málaga, Sierra Tejeda, subida desde El Alcázar, Alcauzín, 29-VI-1978, P. Cubas *et al.* (MA); Jaén, Pontones, barranco del arroyo de las Grajas, 9-V-1981, C. Soriano (MA); Jaén, Cazorla, falda del cerro de la Torquilla, 21-VI-1975, González Rebollar *et al.* (MA); Jaén, Cazorla, aledaños de la C.F. Fuente del Oso, 31-V-1976, González Rebollar *et al.* (MA); Jaén, Cazorla, aledaños de la C.F. Fuente del Oso, 19-VI-1975, González Rebollar *et al.* (MA); Jaén, Cazorla, aledaños de la C.F. Fuente del Oso, 31-V-1976, F. Muñoz Garmendia & C. Soriano (MA).

L. viscosa* subsp. *viscosa

Portugal: Baixo Alentejo, entre Grandola e Alcacer do Sal, 28-IV-1959, Malato-Beliz *et al.* (MA); Baixo Alentejo, entre Figueira de Cavaleiros e Sta. Margarida do Sado, 17-IV-1956, Malato-Beliz *et al.* (MA); Baixo Alentejo, Sines, 10-IV-1946, Bento Rainha (MA).

Spain: Huelva, Parador de Mazagón, 15-II-2006, E. Sánchez-Gullón (MA); Huelva, Matalascañas, 16-III-2009, M. Fernández-Mazuecos & J.L. Blanco 1MF09 (MA); Huelva, Lucena del Puerto, 27-III-2008, E. Sánchez-Gullón (MA); Huelva, Puerto de Santa María, 14-III-2009, J.L. Blanco-Pastor 11JLB09 (MA); Huelva, Enebrales de Punta Umbría, 17-IV-2006, E. Sánchez-Gullón (MA); Huelva, Las Palmeritas, Isla Cristina, 10-IV-2008, E. Sánchez-Gullón (MA); Huelva, Marismas del Odiel, El Almendral, 17-III-2009, M. Fernández-Mazuecos *et al.* 6MF09 (MA); Cádiz, NNW of Algeciras, between estación de Castellar and Malmorailla, 22-IV-1970, V.H. Heywood *et al.* 617 (RNG); Sevilla, road

from Sevilla to Granada between el Arahal and Osuna, 9-IV-1972, D. Bramwell *et al.* (RNG); Sevilla, c. 10 mi. S of Sevilla, between Puebla del Río and Aznalcázar, 30-III-1969, V.H. Heywood *et al.* 385 (RNG); Cádiz, sandy meadow by beach between Hotel Cortijo de la Plata and Zahara de los Atunes, 12-IV-1970, C.R. Fraser-Jenkins (RNG); Cádiz, between Arcos de la Frontera and Bornos on C.342, 22-IV-1973, E.A. Leadlay *et al.* (RNG); Huelva, a 5 km de Ayamonte, 6-IV-1979, M.J. Díez *et al.* (RNG); Almonte, Matalascañas, prov. d'Huelva, 1-III-1977, B. Cabezudo (RNG); Huelva, c. 20 mi. S of La Palma, near El Rocío, 1-IV-1969, V.H. Heywood *et al.* 484 (RNG); Medina Sidonia, prov. Cadiz, Baetica, 18-IV-1925, E. Gros (RNG); Chiclana, Cádiz, 6-V-1967, J. Borja (RNG); Huelva, Villarrasa, junto autopista A-49, 13-IV-1996, Camuñas *et al.* (RNG); Cádiz, Alcalá de los Gazules, Peña del Amor, 13-V-1971, B. Cabezudo *et al.* 278/71 (RNG); Valencia, Carcaixent, 24-IV-1986, G. Mateo *et al.* (MA); Valencia, Carcagente, 24-IV-1986, G. Mateo & S. Piera (MA); Sevilla, Puebla del Río, cerca de la Isla Mayor, 17-III-1968, E.F. Galiano *et al.* (MA); Cádiz, carretera Arcos de la Frontera – Bornos, c. 1 km antes de Bornos, 8-5-1988, F. Muñoz Garmendia & J. Pedrol 2897JP (MA); Cádiz, Algeciras, subiendo al puerto de la Higuera, 25-V-1988, A. Izuzguiza *et al.* 1357AI (MA); Cádiz, Benalup de Sidonia, 10-V-1972, A. González & G. López 1973 GF (MA); Huelva, Matalascañas, pr. Camping 'El Rocío Playa', 12-V-2006, A. Quintanar AQ1900 (MA); Huelva, Matalascañas, pr. Camping 'El Rocío Playa', 12-V-2006, A. Quintanar AQ1903 (MA); Huelva, pr. Almonte, 12-V-2006, A. Quintanar AQ1953 (MA); Huelva, Gibraleón, 17-III-1968, J. Borja (MA); Almonte, Doñana, 20-IV-1977, S. Castroviejo *et al.* 637 SC (MA); Cartaya, Huelva, 12-V-1942, C. Vicioso (MA); Sierra del Endrinal, prope Grazalema, prov. Cádiz, Baetica, 10-VII-1925, E. Gros (MA); Medina Sidonia, prov. Cádiz, Baetica, 18-IV-1925, E. Gros (MA); Málaga, entre Grazalema y Ronda, 23-V-1966, F. Getliffe *et al.* (MA); Sevilla, Coria del Río, 9-III-1968, E.F. Galiano & S. Silvestre (MA).

Fig. S1. Bayesian phylogenetic analysis of ITS haplotypes inferred by PHASE. The fifty-percent majority-rule consensus tree is shown. Numbers above branches are Bayesian posterior probabilities.

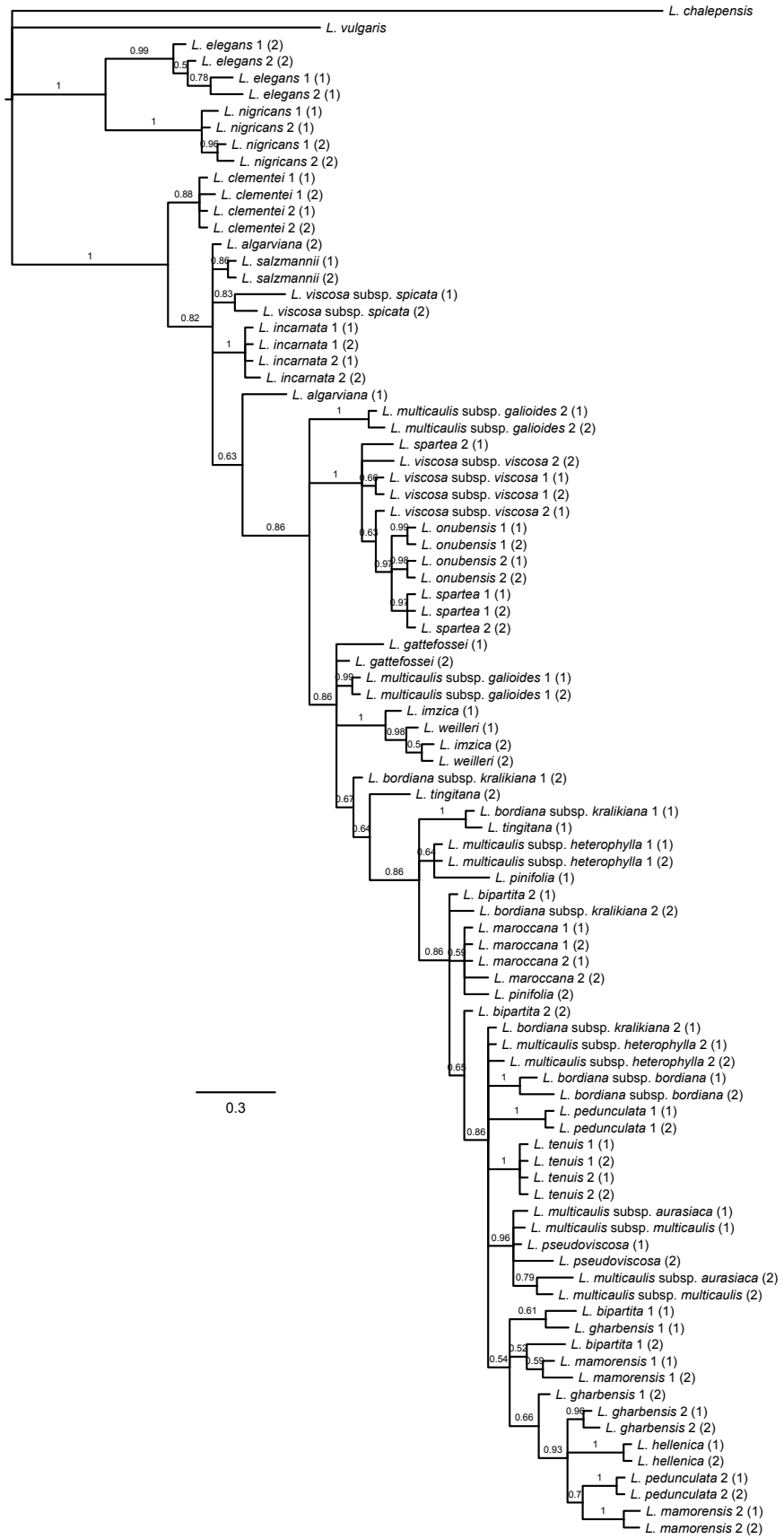


Fig. S2. Maximum clade credibility trees produced by relaxed molecular-clock analyses of ITS (A) and cpDNA (B) sequences in BEAST. Sequences of one individual per taxon of *Linaria* sect. *Versicolores* were included. Node bars represent the 95% highest posterior density intervals for the divergence time estimates of clades with posterior probabilities above 0.50. Values above branches indicate Bayesian posterior probabilities.

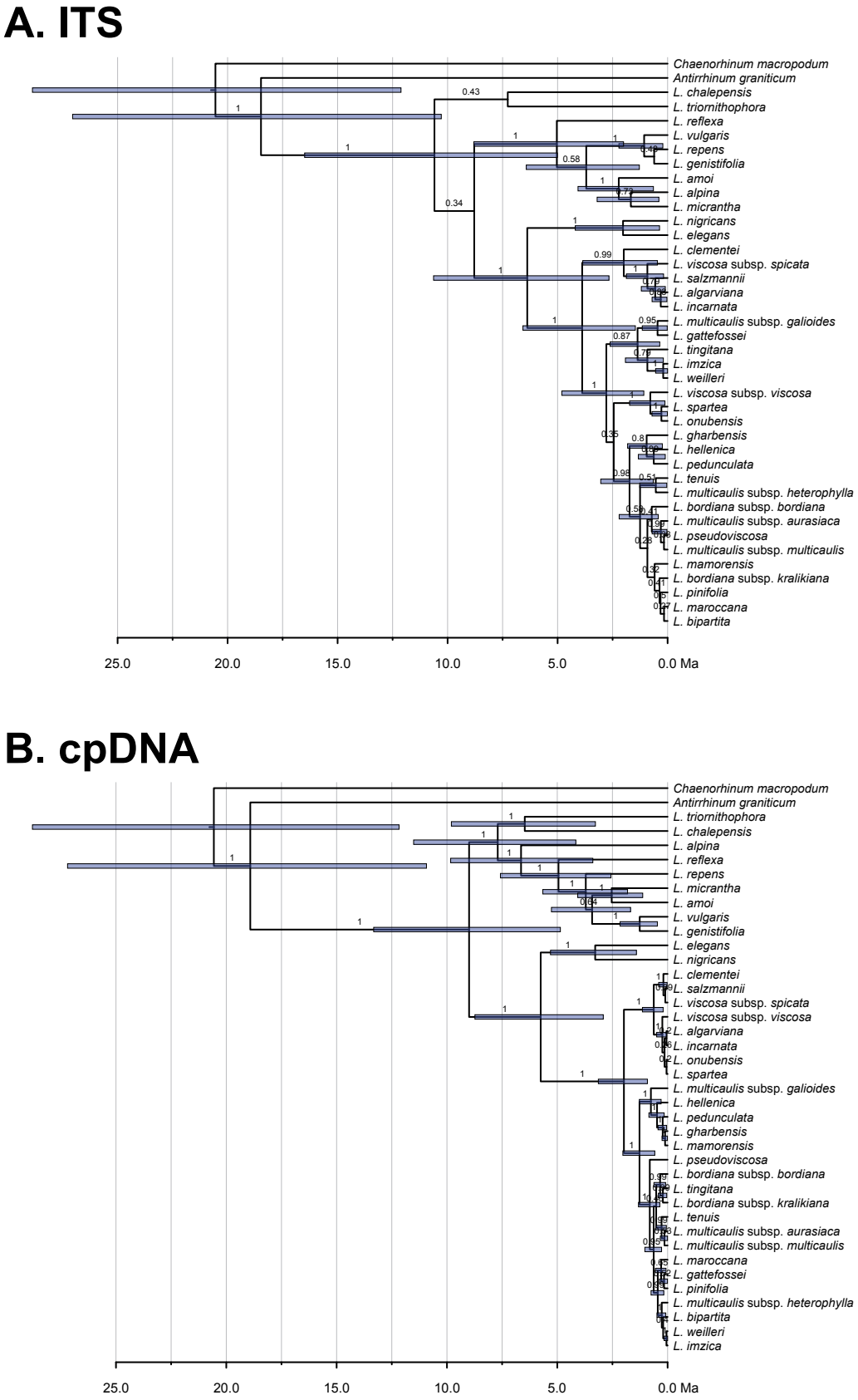


Fig. S3. Scatter plots of tube width *versus* spur length for the 30 species and subspecies of *Linaria* sect. *Versicolores*. The red line separates morphological Types I/II and III.

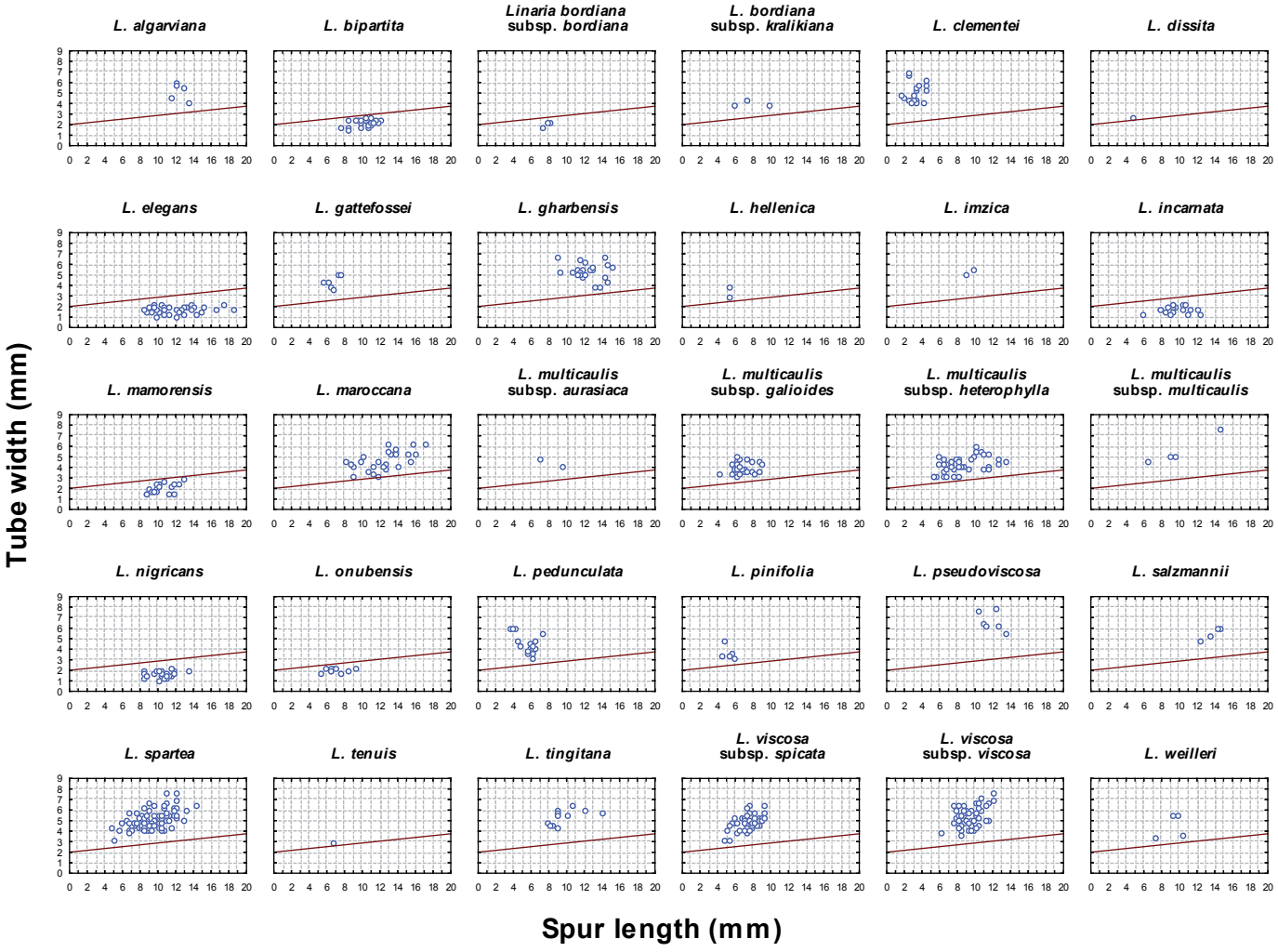


Fig. S4. Scatter plot of tube width *versus* spur length measured from living specimens of the 12 Iberian species and subspecies of *Linaria* sect. *Versicolores*. The three major morphological types discussed in the text are indicated.

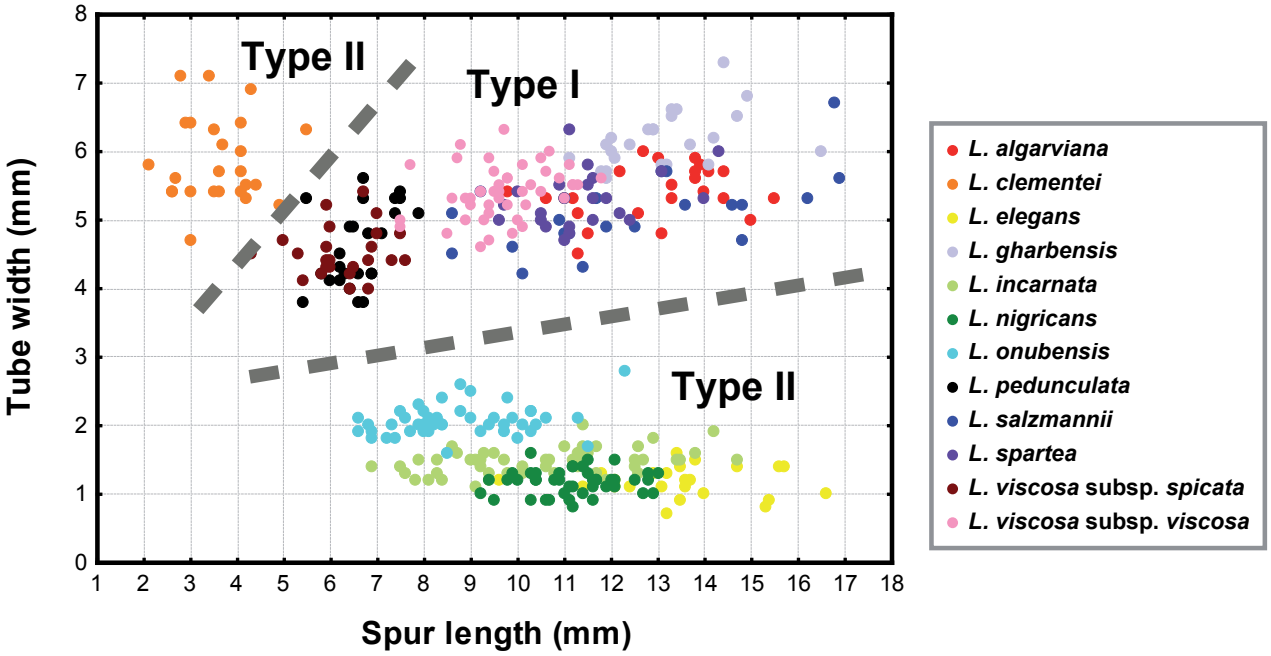


Fig. S5. Visualization of landmark displacements along canonical variates 1 (A) and 2(B) (see Fig. 6).

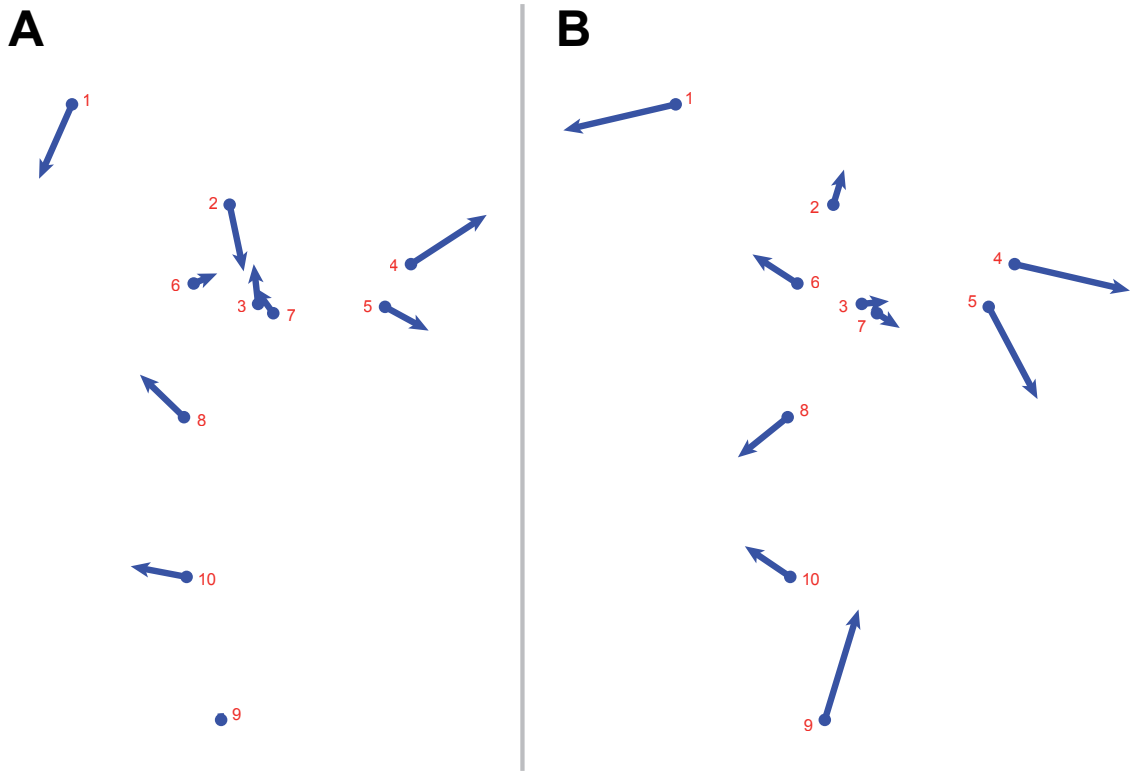


Fig. S6. Results of the binary-state speciation and extinction (BiSSE) analysis of ten species trees randomly chosen from the posterior distribution of the *BEAST analysis. Two character states are considered, which correspond to morphological Types I/II and III. Each row shows the posterior distributions of parameters obtained in the MCMC-BiSSE analysis of one tree. Horizontal bars indicate the 95% credibility interval for each parameter.

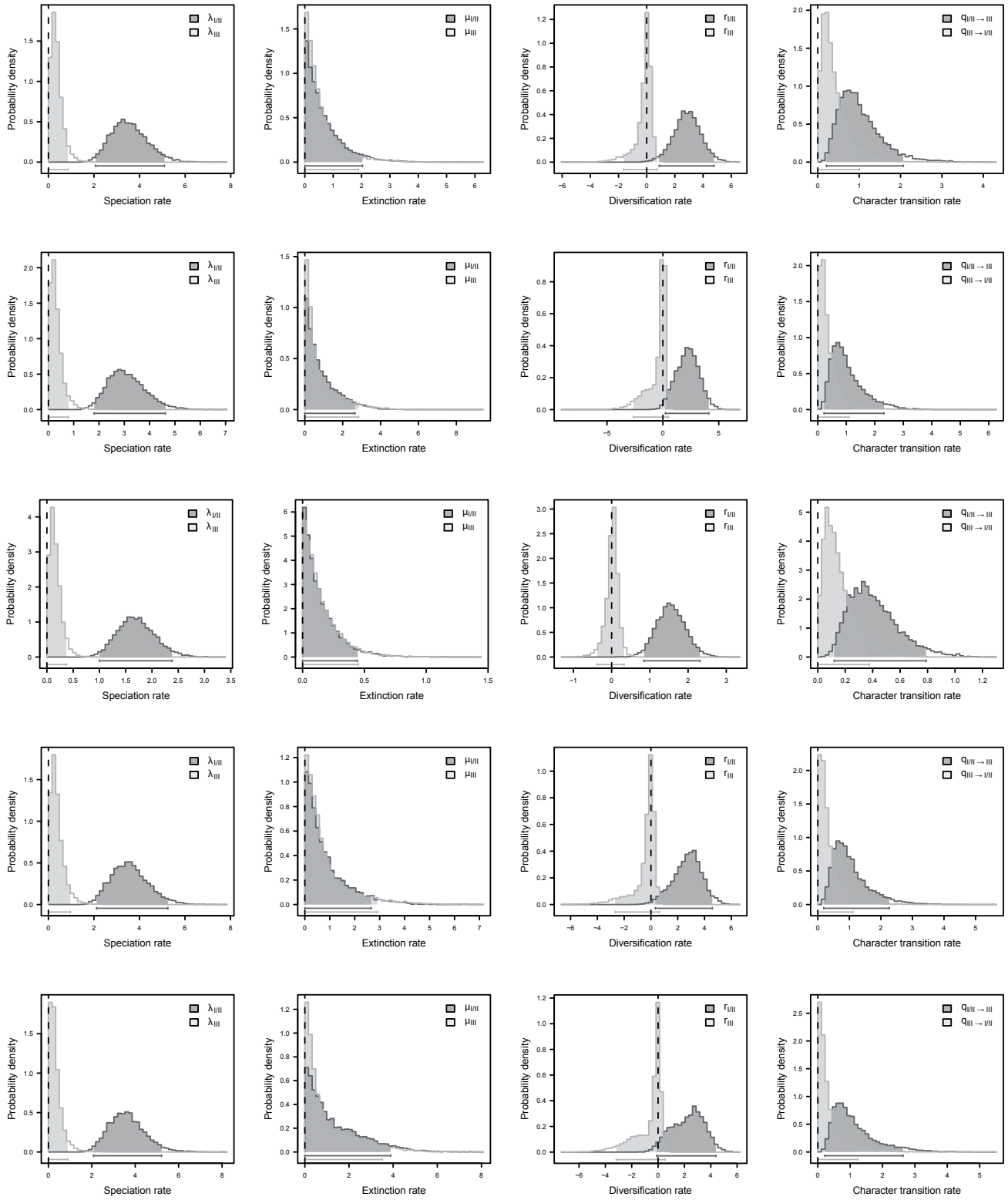


Fig. S6. Continued.

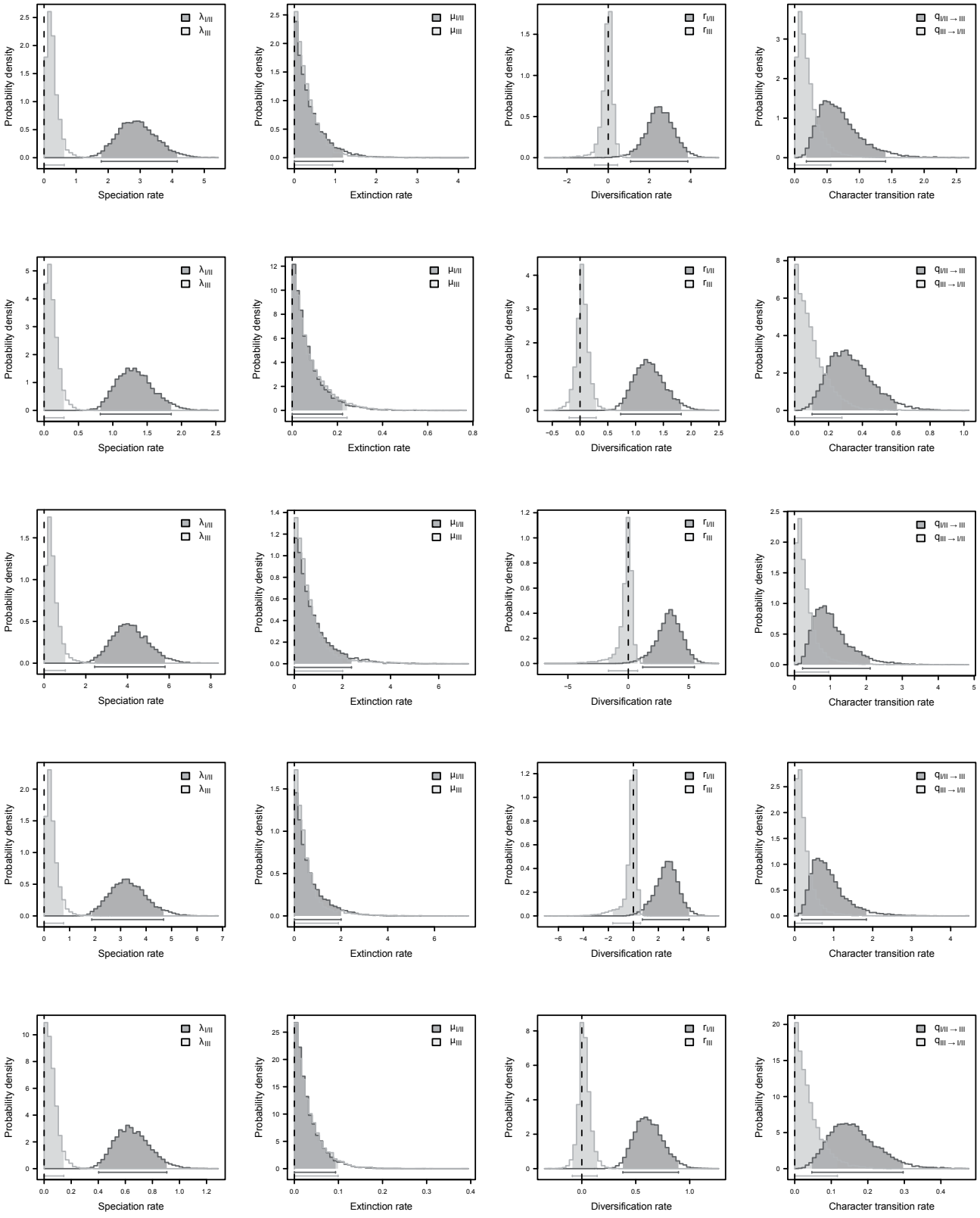


Fig. S7. Ancestral state reconstructions of morphological Types I/II (white) and III (black) under state-dependent diversification (ASR-BiSSE), performed on ten trees randomly chosen from the posterior distribution of the *BEAST analysis. Reconstructions are based on parameter estimates for each tree shown in Fig. S6.

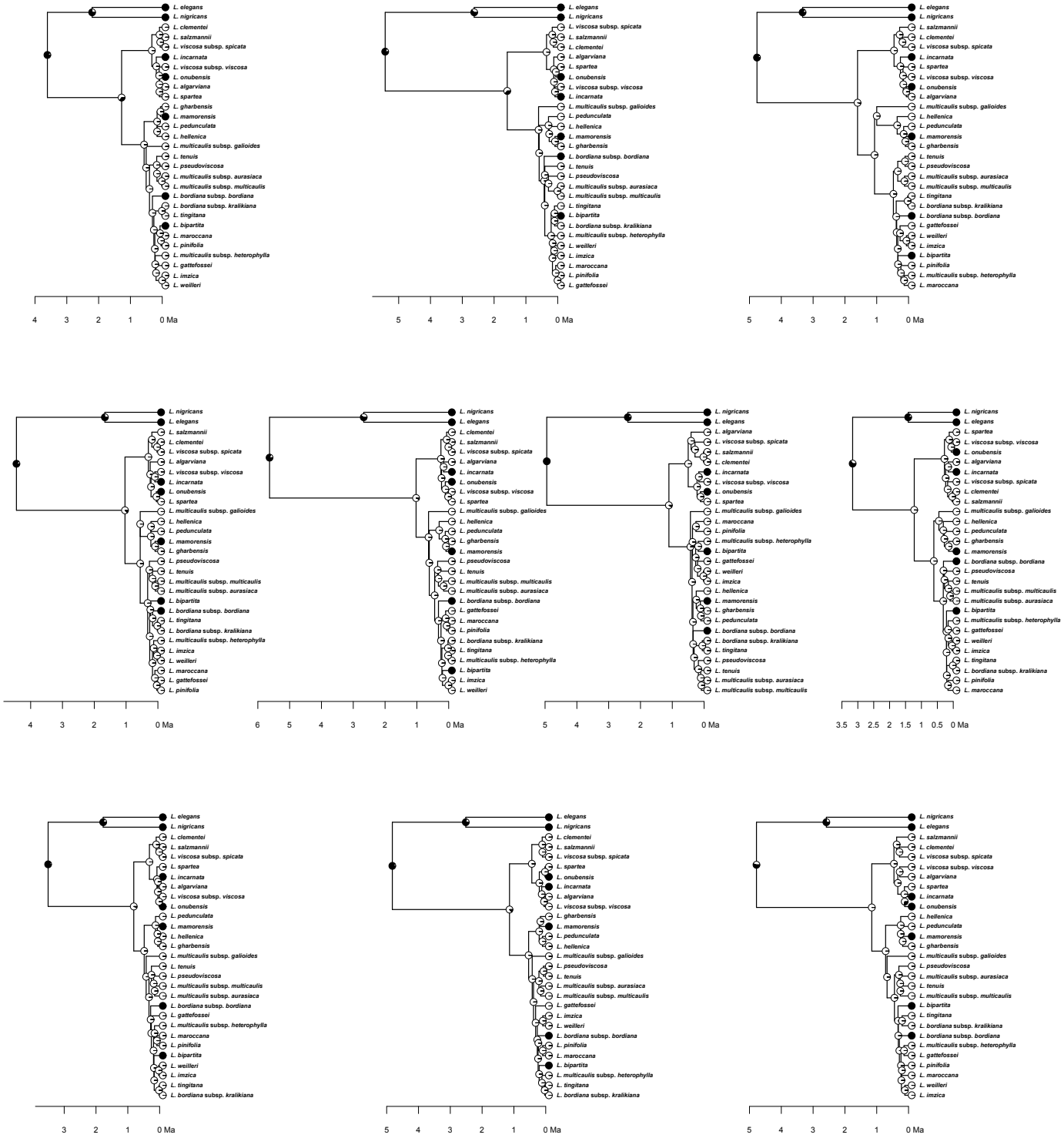


Table S1. Voucher specimens of species and subspecies of *Linaria* sect. *Versicolores* and the outgroup sampled for sequencing of nuclear (ITS) and plastid (*rpl*32-*trn*L^{UAG}, *trn*K-*mat*K, *trn*S-*trn*G) DNA regions.

Taxon	Sampled locality	Voucher
Antirrhinum L.		
Antirrhinum graniticum Rothm.	Spain, Cáceres, Trujillo	P. Vargas 213PV06 (MA)
Chaenorhinum (DC.) Rchb.		
Chaenorhinum macropodum (Boiss. & Reut.) Lange	Spain, Málaga, Cómpeta	P. Vargas 27PV08 (MA)
Linaria Mill.		
Linaria sect. *Diffusae* (Benth.) Wettst.		
L. reflexa (L.) Chaz.	Algeria, Algiers	J.J. Aldasoro A9799 (MA)
Linaria sect. *Linaria*		
L. vulgaris Mill.	France, Chamonix	B. Estébanez s.n. (MA)
Linaria sect. *Macrocentrum* D.A.Sutton		
L. chalepensis (L.) Mill.	Cyprus, Larnaca, Cape Kiti	Iter Mediterraneum IV 294 (MA)
Linaria sect. *Pelisserianae* Valdés		
L. triornithophora (L.) Willd.	Spain, Cáceres, Puerto de Perales	M. Fernández-Mazuecos 18MF07 (MA)
Linaria sect. *Speciosae* (Benth.) Wettst.		
L. genistifolia (L.) Mill.	Turkey, Hadim-Bezkir	J.J. Aldasoro y M.L. Alarcón A9751 (MA)
L. repens (L.) Mill.	Spain, Cuencia, Beteta	M. Fernández-Mazuecos 54MF09 (MA)
Linaria sect. *Supinae* (Benth.) Wettst.		
L. alpina (L.) Mill.	Spain, Huesca, Bujaruelo	J. Güemes s.n. (MA)
L. amoi Campo ex Amo	Spain, Málaga, Cómpeta	M. Fernández-Mazuecos, A.D. Forrest & P. Vargas 30PV08 (MA)
L. micrantha (Cav.) Hoffmanns. & Link	Spain, Alicante, Vall de Gallinera	J.X. Soler & M. Signes 1530-JXS (MA)
Linaria sect. *Versicolores* (Benth.) Wettst.		
Subsect. *Versicolores*		
L. algarviana Chav.	Portugal, Cabo de São Vicente	M. Fernández-Mazuecos 11MF09 (MA)
L. bipartita (Vent.) Willd.	(1) Morocco, Rabat (2) Morocco, Tamri	S.L. Jury with R.G. Wilson 18558 (RNG) S.L. Jury, B. Tahmi & T.M. Upson 14293 (RNG)
L. bordiana Santa & Simonneau		
subsp. *bordiana*	Algeria, Kristel to Aïn Franin	D.A. & S.J. Sutton 248 (RNG)
subsp. *kralikiana* (Maire) D.A.Sutton	(1) Algeria, Mostaganem – Tenes (2) Algeria, Sidi Lakhdar	Davis 51833 (RNG) D.A. & S.J. Sutton 172 (RNG)
L. clementei Haensel. ex Boiss.	(1) Spain, Málaga, Alhaurín de la Torre (2) Spain, Málaga, Coín	M. Fernández-Mazuecos, A.D. Forrest & P. Vargas 7MF08 (MA) M. Fernández-Mazuecos & J. Ramírez 24MF09 (MA)
L. dissita Pomel	-	-
L. gattefossei Maire & Weiller	Morocco, Beni Mellal, El Ksiba	A. Quintanar *et al.* AQ2025 (MA)

Table S1. Continued.

L. gharbensis Batt. & Pit.	(1) Morocco, Rabat (2) Spain, Huelva, Gibraleón	S.L. Jury & R.G. Wilson 18559 (RNG) M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 7MF09 (MA)
L. hellenica Turrill	Greece, Kambos	Unknown collector (ATH)
L. imzica Gómiz	Morocco, Jbel Imzi	F. Gómiz s.n. (MA)
L. incarnata (Vent.) Spreng.	(1) Spain, Badajoz, Alburquerque (2) Spain, Salamanca, Pelabravo	M. Fernández-Mazuecos 9MF09 (MA) M. Fernández-Mazuecos & P. Vargas 39MF09 (MA)
L. maroccana Hook.f.	(1) Morocco, Imouzzer Valley (2) Morocco, Marrakech – Tizi-n-Test	M. Ait Lafkih s.n. (RNG) S.L. Jury, B. Tahiri & T.M. Upson 14209 (RNG)
L. multicaulis (L.) Mill.		
subsp. *multicaulis*	Italy, Sicily, Etna	I. Álvarez *et al.* IA1622 (MA)
subsp. *aurasiaca* (Pomel) D.A.Sutton	Tunisia, El Kesra	Davis & Lamond 57154 (RNG)
subsp. *galioides* (Ball) D.A.Sutton	(1) Morocco, Oukaimedem (2) Morocco, Yagour	P. Jiménez Mejías, E. Narbona, A.J. Chaparro & M. Parra 200PJM05 (UPOS) A. Kool with H.J. Boer, P. Domínguez 904 (RNG)
subsp. *heterophylla* (Desf.) D.A.Sutton	(1) Morocco, Azrou (2) Morocco, Jbel Tazekka	M. Fernández-Mazuecos & J.C. Moreno 15MF08 (MA) E. Rico, S. Andrés & M. Santos SA-221 (SALA)
L. onubensis Pau	(1) Spain, Huelva, Niebla (2) Spain, Huelva, Valverde del Camino	E. Sánchez-Gullón s.n. (MA) V. Valcarcel & P. Vargas 5PV08 (MA)
L. pedunculata (L.) Chaz.	(1) Spain, Almería, Cabo de Gata (2) Spain, Huelva, Marismas del Odiel	M. Fernández-Mazuecos 30MF09 (MA) M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 4MF09 (MA)
L. pinifolia (Poir.) Thell.	Tunisia, El Kala	A. Dubuis, H. Maurel & R. Rhamoun s.n. (RNG)
L. pseudoviscosa Murb.	Tunisia, El Haouaria	P. Wilkin & E.J. Wellens 231 (RNG)
L. salzmannii Boiss.	Spain, Málaga, El Chorro	M. Fernández-Mazuecos & J. Ramírez 19MF09 (MA)
L. spartea (L.) Chaz.	(1) Spain, Madrid, Colmenar (2) Spain, Soria, Tardelcuende	P. Vargas 101PV07 (MA) M. Fernández-Mazuecos, A. Quiroga, S.C. Herrera & D. Orgaz 14MF07 (MA)
L. mamorensis Mazuecos, Vigalondo & L.Sáez	(1) Morocco, Kenitra-Khemisset (2) Morocco, Salé	S. Martín-Bravo, I. Pulgar, F.J. Fernández, G.C. Mazo 34SMB06 (MA) J. Lambinon & G. van den Sande nº95/Ma/333 (RNG)
L. tenuis (Viv.) Spreng.	(1) Libya, Gebel Nefoussa (2) Libya, Tripoli	Davis 49632 (RNG) Davis & Boulos 50581 (RNG)
L. tingitana Boiss. & Reut.	Algeria, El Macta	D.A. & S.J. Sutton 383 (RNG)
L. viscosa (L.) Chaz.		
subsp. *viscosa*	(1) Spain, Huelva, Marismas del Odiel (2) Spain, Huelva, Matalascañas	M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 6MF09 (MA) M. Fernández-Mazuecos & J.L. Blanco 1MF09 (MA)
subsp. *spicata* (Coutinho) D.A.Sutton	Spain, Málaga, Canillas del Aceituno	M. Fernández-Mazuecos & J.L. Blanco 28MF09 (MA)
L. weilleri Emb. & Maire	Morocco, Tirhmi	Miller, Russell & Sutton s.n. (RNG)
Subsect. Elegantes (Viano) D.A.Sutton		
L. elegans Cav.	(1) Spain, Madrid, Puerto de Canencia (2) Spain, Orense, San Xoán de Río	M. Fernández-Mazuecos 30MF08 (MA) M. Fernández-Mazuecos 45MF08 (MA)
L. nigricans Lange	(1) Spain, Almería, Cabo de Gata (2) Spain, Almería, Tabernas	M. Fernández-Mazuecos 29MF09 (MA) P. Vargas 3PV08 (MA)

Table S2. Voucher specimens of Iberian species and subspecies of *Linaria* sect. *Versicolores* and the outgroup sampled for geometric morphometric analyses.

Taxon	Sampled locality	Voucher	No. individuals
L. algarviana	Portugal, Cabo de São Vicente	M. Fernández-Mazuecos 11MF09 (MA)	24
L. clementei	Spain, Málaga, Alhaurín de la Torre	M. Fernández-Mazuecos, A.D. Forrest & P. Vargas 7MF08 (MA)	21
L. elegans	Spain, Madrid, Puerto de Canencia	M. Fernández-Mazuecos 30MF08 (MA)	17
L. elegans	Spain, Portugal, Manteigas	M. Fernández-Mazuecos 127MF10 (MA)	7
L. gharbensis	Spain, Huelva, Gibraleón	M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 7MF09 (MA)	23
L. incarnata	Spain, Badajoz, Alburquerque	M. Fernández-Mazuecos 9MF09 (MA)	24
L. incarnata	Spain, Salamanca, Pelabravo	M. Fernández-Mazuecos & P. Vargas 39MF09 (MA)	24
L. nigricans	Spain, Almería, Cabo de Gata	M. Fernández-Mazuecos 29MF09 (MA)	21
L. nigricans	Spain, Almería, Tabernas	P. Vargas 3PV08 (MA)	22
L. onubensis	Spain, Huelva, Fuente de la Corcha	M. Fernández-Mazuecos & A. Bañón 28MF10 (MA)	20
L. onubensis	Spain, Huelva, Valverde del Camino	V. Valcarcel & P. Vargas 5PV08 (MA)	25
L. pedunculata	Spain, Almería, Cabo de Gata	M. Fernández-Mazuecos 30MF09 (MA)	10
L. pedunculata	Spain, Huelva, Marismas del Odiel	M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 4MF09 (MA)	17
L. salzmannii	Spain, Málaga, El Chorro	M. Fernández-Mazuecos & J. Ramírez 19MF09 (MA)	20
L. spartea	Spain, Badajoz, Alburquerque	M. Fernández-Mazuecos 10MF09 (MA)	25
L. viscosa subsp. *spicata*	Spain, Málaga, Canillas del Aceituno	M. Fernández-Mazuecos & J.L. Blanco 28MF09 (MA)	24
L. viscosa subsp. *viscosa*	Spain, Huelva, Marismas del Odiel	M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 6MF09 (MA)	22
L. viscosa subsp. *viscosa*	Spain, Huelva, Matalascañas	M. Fernández-Mazuecos & J.L. Blanco 1MF09 (MA)	23

Table S3. Measures of spur length and tube width obtained from herbarium specimens of the 30 species and subspecies of *Linaria* sect. *Versicolores*. Values are shown as means ± standard deviations. Morphological type for each taxon (as discussed in the text) is indicated.

Taxon	Spur length (mm)	Tube width (mm)	Morphological type
Subsect. *Versicolores*			
L. algarviana	12.66 ± 0.72	5.13 ± 0.87	I
L. bipartita	10.09 ± 1.48	2.16 ± 0.33	III
L. bordiana subsp. *bordiana*	7.48 ± 0.77	2.04 ± 0.26	III
L. bordiana subsp. *kralikiana*	8.62 ± 2.35	3.94 ± 0.27	I
L. clementei	3.20 ± 0.81	5.03 ± 0.92	II
L. dissita	5.05 ± 0.24	2.56 ± 0.15	I
L. gattefossei	6.68 ± 0.74	4.32 ± 0.59	I
L. gharbensis	12.24 ± 1.60	5.32 ± 0.76	I
L. hellenica	5.48 ± 0.01	3.24 ± 0.64	I
L. imzica	10.24 ± 1.66	5.30 ± 0.36	I
L. incarnata	9.62 ± 1.39	1.67 ± 0.35	III
L. mamorensis	10.16 ± 1.17	1.93 ± 0.42	III
L. maroccana	12.87 ± 2.49	4.59 ± 0.91	I
L. multicaulis subsp. *aurasiaca*	8.42 ± 1.33	4.39 ± 0.47	I
L. multicaulis subsp. *galioides*	6.76 ± 1.04	3.91 ± 0.49	I
L. multicaulis subsp. *heterophylla*	8.58 ± 2.15	4.25 ± 0.72	I
L. multicaulis subsp. *multicaulis*	10.00 ± 3.47	5.49 ± 1.39	I
L. onubensis	6.78 ± 1.17	1.97 ± 0.22	III
L. pedunculata	6.02 ± 1.07	4.46 ± 0.85	I
L. pinifolia	5.25 ± 0.65	3.60 ± 0.62	I
L. pseudoviscosa	11.96 ± 1.07	6.61 ± 0.88	I
L. salzmannii	13.47 ± 1.10	5.40 ± 0.61	I
L. spartea	9.41 ± 1.96	5.04 ± 0.85	I
L. tenuis	5.53 ± 1.09	2.80	I
L. tingitana	9.87 ± 1.80	5.32 ± 0.69	I
L. viscosa subsp. *spicata*	7.52 ± 1.24	4.81 ± 0.74	I
L. viscosa subsp. *viscosa*	9.30 ± 1.26	5.35 ± 0.93	I
L. weilleri	9.28 ± 1.41	4.45 ± 1.17	I
Subsect. Elegantes			
L. elegans	12.44 ± 2.53	1.64 ± 0.31	III
L. nigricans	10.39 ± 1.23	1.61 ± 0.30	III

Capítulo 5

Historia de *Linaria elegans* en el Cuaternario reconstruida mediante modelos de distribución de especies y análisis filogeográficos

Congruence between distribution modelling and phylogeographic analyses reveals Quaternary survival of a toadflax species (*Linaria elegans*) in oceanic-climate areas of a mountain ring range

Una versión de este capítulo se encuentra en proceso de revisión para su publicación en *New Phytologist*

Abstract

The role of Quaternary climatic shifts in shaping the distribution of *Linaria elegans*, an Iberian annual plant, is investigated using species distribution modelling and molecular phylogeographic analyses. Three hypotheses are proposed to explain the Quaternary history of its mountain ring range. The distribution of *L. elegans* was modelled using the maximum entropy method and projected to the last inter-glacial and to the last glacial maximum (LGM) using two different paleoclimatic models (CCSM and MIROC). Two nuclear and three plastid DNA regions were sequenced for 24 populations (119 individuals sampled). Bayesian phylogenetic, phylogeographic, dating and coalescent-based population genetic analyses were conducted. Molecular analyses indicated the existence of northern and southern glacial refugia and two routes of post-glacial re-colonization. These results were consistent with the LGM distribution as inferred under the CCSM paleoclimatic model (but not under the MIROC model). Isolation between the two major refugia was dated back to the Riss or Mindel glaciations, c. 300 ka BP. The Atlantic distribution of inferred refugia suggests that the oceanic (buffered) – continental (harsh) gradient may have played a key and previously unrecognized role in determining Quaternary distribution shifts of Mediterranean plants.

Introduction

The climatic changes of the Quaternary (i.e., the last 2.6 Ma) have shaped the distribution and abundance of extant species in a variety of ways (Bennett & Provan, 2008; Stewart *et al.*, 2010). Among temperate species, these climatic shifts have brought about several cycles of contraction and expansion of their geographical ranges. Certain regions acted as refugia for these species during the ice ages, from where they were able to recolonize previously occupied areas during inter-glacial periods (Hewitt, 1996; Comes & Kadereit, 1998; Hewitt, 2000). In particular, the three Mediterranean peninsulas of Southern Europe (Iberian, Italian and Balkan) are known to have acted as refugia during the Quaternary. They remained mostly ice-free during the last glacial maximum, i.e. 26.5 to 19–20 ka before present (BP) (Clark *et al.*, 2009), harbouring many European species and enabling the recolonization of central and northern Europe in the post-glacial period (Comes & Kadereit, 1998; Taberlet *et al.*, 1998; Hewitt, 1999; Schmitt, 2007). Recently, however, the historical complexity of these three peninsulas has been recognized with the demonstration that they did not constitute single refugia throughout the Quaternary but instead several independent or interconnected "refugia within refugia" (Gómez & Lunt, 2006; Nieto-Feliner, 2011). It has also been argued that altitudinal migration, rather than large-scale range shifts, may have dominated the Quaternary history of Mediterranean species (Gutiérrez-Larena *et al.*, 2002; Martín-Bravo *et al.*, 2010). Still, different species are adapted to tolerate different ranges of climatic conditions and therefore may respond to climatic shifts in diverse ways (Stewart *et al.*, 2010). Considering the high species diversity in the Mediterranean region, yet the low number of species studied to date, further research is needed to understand the Quaternary history of Mediterranean taxa.

Three main approaches are employed to reconstruct species ranges throughout the Quaternary climatic cycles: (1) the study of the spatial and temporal distribution of macro- and microfossils; (2) the analysis of DNA markers in living populations to reconstruct phylogeographic patterns; and (3) the projection of species distribution models (SDMs) to past climatic conditions. These approaches have been extensively used to study the distribution range of plants, including Mediterranean taxa, in the last few years (e.g. Carrión *et al.*, 2003; Benito Garzón *et al.*, 2007; López de Heredia *et al.*, 2007; Picó *et al.*, 2008; Calleja *et al.*, 2009; González-Sampériz *et al.*, 2010; Désamoré *et al.*, 2012). However, they have been rarely integrated (but see Rodríguez-

Sánchez & Arroyo, 2008; Rodríguez-Sánchez *et al.*, 2009 for an example with the laurel tree), even though combined analyses using different data sources is highly desirable in this field (Taberlet *et al.*, 1998). Particularly in the case of herbaceous taxa, the paleobotanical approach has been seldom implemented, as these plants are only rarely registered in the fossil record. The reconstruction of past distribution ranges of herbs has therefore relied on the investigation of their phylogeographic patterns and the projection of SDMs, two approaches that can provide complementary and meaningful information when combined (Waltari *et al.*, 2007; Chan *et al.*, 2011).

Linaria elegans Cav. is an Iberian, annual species of *Linaria* section *Versicolores*, a monophyletic assemblage within the largest genus of the snapdragon tribe (Antirrhineae) (Sutton, 1988; Chapter 2; Appendix 2). It is an allogamous, insect-pollinated toadflax (Fernández-Mazuecos *et al.*, unpublished results), with a diploid chromosome number of 2n = 12 (Heitz, 1926). Divergence between *L. elegans* and its sister species *L. nigricans* has been dated back to the early Pliocene–early Pleistocene (Fernández-Mazuecos & Vargas, 2011b; Chapter 3), thus making *L. elegans* an appropriate species for the investigation of distribution shifts associated with the Quaternary climatic cycles. The current distribution of *L. elegans* encompasses a diverse range of habitats, such as siliceous sandy soils up to 1900 m in therophytic communities, open scrubs and woodlands, as well as open habitats as low as 100 m in oceanic areas (Sáez & Bernal, 2009). It is primarily distributed in a discontinuous mountain ring surrounding the northern plateau of Iberia (Duero river basin). The observation of such a distribution pattern raises multiple questions about its origin and the extent to which it was shaped by climatic oscillations. Three main hypotheses can be considered to explain the Quaternary history of mountain ring distributions (Fig. 1). First (Hypothesis I), the cold temperatures of glacial periods may have caused an altitudinal descent of populations, which may have then expanded their distribution across the central basin, leading to the admixture of populations from different ranges of the mountain ring, followed by post-glacial centrifugal recolonization of the ring (e.g. Robledo-Arnuncio *et al.*, 2005). A second hypothesis (Hypothesis II) is that cycles of altitudinal descent have occurred without an admixture of populations from different mountains, leading to long-term isolation between areas (similar to Ronikier *et al.*, 2008). Finally (Hypothesis III), the glacial periods may have caused extinction of populations and a contraction of the distribution range into a few favourable environments (refugia), found most likely (although maybe not

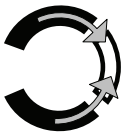
	LAST INTER-GLACIAL (LIG)	LAST GLACIAL MAXIMUM (LGM)	POST-GLACIAL RECOLONIZATION	PRESENT	PHYLOGEOGRAPHIC PREDICTIONS
HYPOTHESIS I. Altitudinal-descent migration with admixture					**1.** All population splits between mountain ranges postdate the LGM. **2.** Absence of linear spread patterns along the mountain range.
HYPOTHESIS II. Altitudinal-descent migration without admixture					**1.** All population splits between mountain ranges predate the LGM. **2.** Absence of linear spread patterns along the mountain range.
HYPOTHESIS III. Restricted glacial refugia					**1.** Population splits between different refugia may have predated or coincided with the LGM. Splits between refugia and recolonized areas postdate the LGM. **2.** Linear spread inferred along the mountain ring.

Fig. 1. Testable hypotheses about the late Quaternary history of a mountain ring species. The ring represents a mountain ring that surrounds an inner basin, with each segment symbolizing a distinct mountain area. Areas filled in black represent those occupied by the species during the LIG, LGM and present time according to each hypothesis. Grey arrows indicate routes of post-glacial colonization. Predictions concerning phylogeographic patterns are outlined for each hypothesis.

exclusively) in the south (Comes & Kadereit, 1998). In this case, the ring distribution observed nowadays would be the result of post-glacial recolonization from glacial refugia along the mountain ring. Notice that all three hypotheses assume that the distribution pattern during past inter-glacial periods was a ring similar to that presently observed. Phylogeographic patterns predicted under the three hypotheses are summarized in Fig. 1. These predictions can be tested thanks to recently-developed Bayesian phylogeographic methods, which are capable to estimate split times between populations (Hey, 2010) and directions of spread (Lemey *et al.*, 2009).

In this study, we discriminated between the three hypotheses outlined above as applied to the late Quaternary history of *L. elegans*. To this end, we integrated a time-calibrated phylogeographic analysis of *L. elegans* based on plastid and nuclear markers with species distribution modelling under current, last glacial maximum (LGM) and last inter-glacial (LIG) climatic conditions.

Materials and methods

Mountain ring distribution

The current distribution of *L. elegans* in the northern half of the Iberian Peninsula was divided into nine areas (Fig. 2). Seven areas were placed along the mountain ring that surrounds the Duero basin: Cantabrian Mountains (CM), Galician-Portuguese Mountains (GP), Serra da Estrela (ES), Sierra de Gata (GA), Sierra de Gredos (GR), Sierra de Guadarrama (GU) and Northern Iberian System (NI). We defined two additional peripheral areas outside the mountain ring: Atlantic lowlands (AL) in the northwest, and Southern Iberian System (SI) in the southeast. In addition, we defined three major regions encompassing the nine areas: Cantabrian region in the north (CM, GP, AL), Central Mountain System (henceforth Central System) in the south (ES, GA, GR, GU), and Iberian Mountain System (henceforth Iberian System) in the east (NI, SI).

Species distribution modelling

Species distribution modelling (SDM) was performed to evaluate the potential distribution of *L. elegans* under present climatic conditions and to project it to the past. We employed the maximum entropy algorithm, as implemented in Maxent v3.3 (Phillips *et al.*, 2006) because it is appropriate for presence-only data and its performance has been shown to be consistently competitive in comparison with other methods (Elith *et al.*, 2006). We retrieved a set of 19 bioclimatic variables under current conditions from the WorldClim website (www.worldclim.org; Hijmans *et al.*, 2005). GIS layers were clipped to the extent of the Iberian Peninsula. After a correlation analysis in a random sample of 1000 points of the Iberian Peninsula, we selected a minimum set of seven uncorrelated variables (Table 1). Given that *L. elegans* is a silicicolous (acidophilous) species (Sáez & Bernal, 2009), we also included a categorical variable distinguishing acidic and basic substrates. This layer was derived from the dominant parent material layer of the European Soil Database (Panagos, 2006).

The eight variables were used as predictors to calibrate the distribution model in Maxent. In the occurrence dataset, we included 78 point localities obtained from our own collections, specimens of the MA herbarium, the Spanish plants information system *Anthos* (Castroviejo *et al.*, 2006) and additional bibliographic and on-line sources (Fig. 2; Supporting Information Table

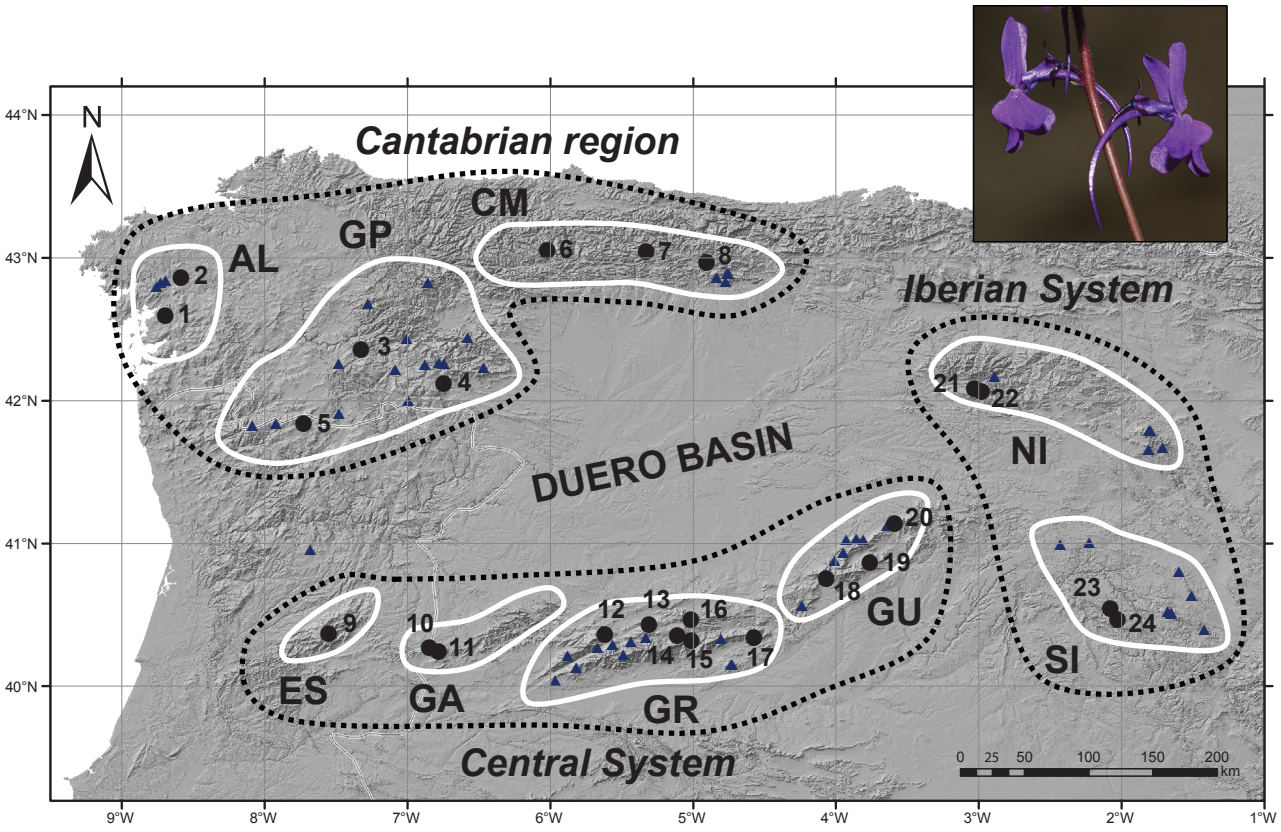


Fig. 2. Geographic distribution of the 24 populations of *Linaria elegans* sampled for phylogeographic analyses (numbered dots, see Table 2) and the 54 localities additionally employed for distribution modelling (triangles, see Supporting Information Table S1). The three major regions of *L. elegans* distribution (Cantabrian region, Central System and Iberian System) are indicated, as well as the smaller areas delimited for phylogeographic reconstructions: Cantabrian Mountains (CM), Galician-Portuguese Mountains (GP), Atlantic lowlands (AL), Serra da Estrela (ES), Sierra de Gata (GA), Sierra de Gredos (GR), Sierra de Guadarrama (GU), Northern Iberian System (NI) and Southern Iberian System (SI). The inset shows a photograph of *L. elegans* flowers.

S1). Only reliably identified and accurately geo-referenced (at least 1 km precision) records were considered. When running the algorithm, occurrence data were randomly split into training data (75%), used for model building, and test data (remaining 25%), used to evaluate model accuracy. Ten subsample replicates were performed, and fitness of the resulting model was assessed with the area under the receiver-operating characteristic (ROC) curve (Phillips *et al.*, 2006). A jackknife analysis was employed to evaluate variable contributions to the model, and response curves were created to assess to which extent the predictions were affected by variable values. To convert continuous suitability values to presence/absence data, we chose

Table 1. Seven uncorrelated bioclimatic variables employed for distribution modelling of *L. elegans* in Maxent. Mean value and range for each variable in the studied area are shown under current conditions, as well as LGM (CCSM and MIROC) and LIG simulations.

Name	Variable	Current mean [min – max]	LGM-CCSM mean [min – max]	LGM-MIROC mean [min – max]	LIG mean [min – max]
bio3	Isothermality (%)	37.7 [26 – 52]	45.5 [32 – 58]	36.0 [23 – 50]	30.0 [18 – 45]
bio4	Temperature seasonality (ºC)	5.8 [2.6 – 8.0]	5.6 [2.4 – 8.0]	5.6 [2.4 – 8.0]	7.8 [3.5 – 10.8]
bio5	Maximum temperature of warmest month (ºC)	29.1 [9.2 – 40.1]	27.9 [10.4 – 42.1]	26.4 [9.2 – 37.7]	33.3 [14.2 – 44.9]
bio6	Minimum temperature of coldest month (ºC)	2.0 [-12.5 – 9.4]	-2.1 [-18.3 – 8.4]	1.1 [-12.2 – 8.8]	4.9 [-14.4 – 8.2]
bio13	Precipitation of wettest month (mm)	82.2 [15 – 273]	131.2 [16 – 485]	126.7 [32 – 411]	103.1 [25 – 319]
bio14	Precipitation of driest month (mm)	18.6 [0 – 117]	24.4 [1 – 136]	25.2 [1 – 135]	14.5 [0 – 107]
bio15	Precipitation seasonality (mm)	39.3 [11 – 88]	41.0 [11 – 87]	41.0 [11 – 87]	45.0 [15 – 84]

the threshold obtained under the maximum training sensitivity plus specificity rule, which has been shown to produce accurate predictions (Jiménez-Valverde & Lobo, 2007).

The distribution model under current conditions was projected to the last glacial maximum (LGM; c. 21 ka BP) using paleoclimatic layers simulated from two general atmospheric circulation models: the Community Climate System Model (CCSM; Collins *et al.*, 2006) and the Model for Interdisciplinary Research on Climate (MIROC; Hasumi & Emori, 2004). We also projected the model to the last inter-glacial (LIG; c. 120–140 ka BP) using the climatic model of Otto-Bliesner *et al.* (2006). It was assumed that the ecological requirements of *L. elegans* have remained stable throughout at least the last climatic cycle (Nogués-Bravo, 2009), which seems fairly reasonable given the short time scale.

DNA sampling and sequencing

We sampled 119 individuals of *L. elegans* from 24 populations (four or five individuals per population) covering the whole distribution range of the species (Fig. 2; Table 2). Twenty of these populations were sampled along the mountain ring, and four in peripheral areas. All plant material was collected in the field and dried in silica gel. Herbarium specimens were deposited in the MA herbarium.

Procedures used for DNA extraction, amplification and sequencing followed Fernández-Mazuecos & Vargas (2011b; see Chapter 3). First, a pilot study using four geographically distant samples was performed to find consistently amplified and variable DNA regions. We tested six cpDNA regions previously used in phylogeographic analyses: *rpl*32-*trn*L^{UAG}, *trn*Q-*rps*16, *trn*K-*mat*K, *trn*S-*trn*G, *trn*H-*psb*A and *trn*L^{UAA} intron (Johnson & Soltis, 1994; Hamilton, 1999; Shaw *et al.*, 2007; Taberlet *et al.*, 2007; Hollingsworth *et al.*, 2009), all of which were consistently amplified and sequenced. Three regions with the highest nucleotide variation were identified: *rpl*32-*trn*L^{UAG}, *trn*K-*mat*K and *trn*S-*trn*G, which were then sequenced for five individuals per population, except for population 6, where only four individuals were sampled (119 individuals in total). In all cases, the same standard primers were employed for amplification and sequencing.

Four nuclear DNA regions were tested in the pilot study: three of the low-copy phylogeny Conserved Ortolog Set (pCOS) genes developed by Li *et al.* (2008), plus the nuclear ribosomal

Table 2. Populations of *Linaria elegans* sampled for DNA sequencing, indicating haplotypes of the combined cpDNA (*rpl*32-*trn*L^{UAG}/*trn*K-*mat*K/*trn*S-*trn*G) and *At103* datasets (see Fig. 2 for geographic areas).

Geographic area	Population number	Locality	Altitude (m)	cpDNA haplotypes	*At103* haplotypes
AL	1	Spain, Pontevedra, Caldas de Reis	47	G (x5)	2 (x2), 4 (x4), 7 (x2)
	2	Spain, A Coruña, Santiago de Compostela	169	H (x3), I (x2)	2 (x4), 13 (x2)
GP	3	Spain, Ourense, San Xoán de Río	912	B (x2), H (x3)	2 (x2), 5 (x4)
	4	Spain, Zamora, Ribadelago	1012	D, E, F, G, H	2 (x7), 12
	5	Portugal, Montalegre	930	A, H, J (x3)	2 (x5), 4
CM	6	Spain, León, Puerto de Ventana	1473	B (x2), G (x2)	2 (x2)
	7	Spain, León, Isoba	1489	C (x2), G, H (x2)	1 (x2), 2 (x4), 20 (x2)
	8	Spain, León, Villafrea de la Reina	1160	G (x5)	1 (x2), 2 (x2), 21 (x2)
ES	9	Portugal, Manteigas	1046	K (x5)	8 (x2), 15 (x2)
GA	10	Spain, Salamanca, Navasfrías	1014	A (x4), K	-
	11	Spain, Cáceres, Eljas	1062	A (x4), K	2 (x5), 6 (x2), 18 (x2)
GR	12	Spain, Ávila, Puerto del Tremedal	1640	M (x2), Q (x3)	2 (x2), 14, 18, 19 (x2)
	13	Spain, Ávila, Puerto de Peñanegra	1446	L, M, Q, S (x2)	2 (x3), 3 (x2), 11 (x2), 14
	14	Spain, Ávila, Navarredonda de Gredos	1605	L, M (x3), R	4 (x2), 9, 17, 18 (x2)
	15	Spain, Ávila, Puerto del Pico	1385	L (x2), M (x3)	3 (x4)
	16	Spain, Ávila, Puerto de Menga	1555	L, M (x2), Q (x2)	4 (x2)
	17	Spain, Ávila, Puerto de Casillas	1461	L, N, O (x2), P	2 (x5), 22
GU	18	Spain, Madrid, Cercedilla	1293	M, S, T (x3)	2 (x2), 3 (x3), 10, 17, 18 (x2)
	19	Spain, Madrid, Puerto de Canencia	1527	R, S (x3), T	2, 4 (x3), 16 (x2)
	20	Spain, Madrid, Somosierra	1538	S (x2), T (x3)	2 (x2), 4 (x2), 18 (x2), 23
NI	21	Spain, Burgos, Cerro Grañón	1688	S (x5)	2 (x2), 18, 23 (x5)
	22	Spain, Burgos, Neila	1236	S (x5)	2 (x3), 9 (x3), 22 (x2)
SI	23	Spain, Cuenca, El Tobar	1304	S (x5)	2 (x3), 4, 10 (x3), 18
	24	Spain, Cuenca, Santa María del Val	1364	S (x5)	2 (x3), 16 (x2), 18

internal transcribed spacer (ITS1-5.8S-ITS2). The three tested pCOS genes (*Agt1*, *At103* and *Eif3E*) were amplified using primers and PCR protocols described by Li *et al.* (2008) and directly sequenced. We selected the *At103* gene (Rzeznicka *et al.*, 2005) for further sequencing because it provided the highest consistency in amplification and sequencing, as well as high nucleotide variation. Amplification was performed for all 119 sampled individuals. Additionally, the ITS region was sequenced from one individual per population (24 individuals in total), using the following PCR conditions: 1 min pretreatment at 94ºC and 30 cycles of 1 min at 94ºC, 1 min at 56ºC and 1 min at 72ºC. We employed the external primers 17SE and 26SE (Sun *et al.*, 1994) for amplification, and the internal primers ITS5 (Sang *et al.*, 1995) and ITS4 (White *et al.*, 1990) for sequencing.

As the outgroup, sequences of the same plastid and nuclear regions were obtained for two individuals of the sister species *L. nigricans* and two or three individuals of additional species of sect. *Versicolores*: *L. spartea* and *L. gharbensis* for cpDNA regions and ITS; and *L. spartea*, *L. clementei* and *L. pedunculata* for *At103* (Supporting Information Table S2). All sequences were assembled in Geneious Pro (Drummond *et al.*, 2010). Ambiguous nucleotides were represented by IUPAC symbols.

Sequences of each DNA region were separately aligned using MAFFT 6 (Katoh *et al.*, 2002) with default parameters, and further adjustments were made by visual inspection. The three cpDNA regions were concatenated in a single matrix.

Analysis of DNA haplotypes

In order to reconstruct haplotypes from the diploid unphased *At103* sequences, we employed the Bayesian statistical method PHASE 2.1 (Stephens *et al.*, 2001; Stephens & Donnelly, 2003), as implemented in DnaSP v5 (Librado & Rozas, 2009), with default parameters (recombination model MR0, 100 iterations, 100 burn-in iterations, thinning interval 1). Five runs were performed, and the one with the best average goodness-of-fit was selected. Only highly supported haplotype pairs (probability > 0.90) were maintained. We used IMgc (Woerner *et al.*, 2007) to generate a reduced, recombination-free matrix, which would be needed for coalescent-based analyses that assume no recombination (see below). This reduced dataset was used for all subsequent analyses.

The concatenated cpDNA dataset and the recombinantion-free *At103* dataset were analyzed using the statistical parsimony algorithm (Templeton *et al.*, 1992), as implemented in TCS 1.21 (Clement *et al.*, 2000), in order to infer genealogical relationships among haplotypes. The maximum number of differences resulting from single substitutions among haplotypes was calculated with 95% confidence limits, treating gaps as missing data.

Phylogenetic analyses

Relationships among sequences were assessed using Bayesian inference (BI) and maximum parsimony (MP). We conducted separate analyses on the concatenated cpDNA dataset and the recombination-free *At103* dataset, after removing identical sequences from both. For the ITS dataset (for which haplotypes had not been inferred given its multicopy nature and the high number of additive polymorphic sites), all sequences were included. In all analyses, the additional sequenced species of sect. *Versicolores* were included, and *L. spartea* was set as the outgroup based on previous phylogenetic evidence (Fernández-Mazuecos & Vargas, 2011b; Chapter 3; Chapter 2; Appendix 2).

For the BI analyses, models of nucleotide substitution (F81+G for *rpl*32-*trn*L^{UAG}; HKY+Y for *trn*K-*mat*K; GTR+I for *trn*S-*trn*G; HKY+G for *At103* and GTR+I+G for ITS) were selected for each DNA region under the Akaike Information Criterion (AIC) in jModelTest 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). BI was performed in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) using two searches with 10 million generations each and a sample frequency of 1000. In the plastid analysis, the three regions were partitioned, and substitution models were unlinked across partitions. Fifty-percent majority rule consensus trees with Bayesian posterior probabilities (PP) of clades were calculated after removing the first 10% generations as burn-in.

MP analyses were performed in TNT 1.1 (Goloboff *et al.*, 2003). Given the low number of sequences in the cpDNA matrix, the analysis was performed under the implicit enumeration method, and the option "collapse trees after the search" was selected in order to discard unsupported nodes. For the *At103* and ITS datasets, we employed a "traditional search" with 10,000 replicates saving two most-parsimonious trees per replicate, followed by a second heuristic search retaining all best trees and using the trees obtained in the previous 10,000

replicates as the starting ones. Bootstrap support (BS) of clades was assessed for all datasets using 10,000 standard replicates.

Estimation of divergence times

To estimate divergence times among *L. elegans* cpDNA lineages, we combined newly generated sequences of the study species with the *rpl*32-*trn*L^{UAG}/*trn*K-*mat*K sequences of *Linaria* sect. *Versicolores* previously published (Fernández-Mazuecos & Vargas, 2011b; see Chapter 3). Identical sequences of the matrix were removed, treating gaps as missing data. A relaxed molecular-clock approach was implemented in BEAST v.1.6.2 (Drummond *et al.*, 2006; Drummond & Rambaut, 2007) following procedures of Fernández-Mazuecos & Vargas (2011b; see Chapter 3). Since our dataset included inter- and intraspecific data, two different tree priors were tested: a birth-death process (Gernhard, 2008) and a Bayesian skyline plot (Drummond *et al.*, 2005). The two cpDNA regions were treated as different partitions, with unlinked substitution models (GTR+G for *rpl*32-*trn*L^{UAG} and HKY+G for *trn*K-*mat*K). Since no reliable fossils of *Linaria* are known to date we implemented a secondary, basal calibration. The divergence time between *Chaenorhinum* and *Linaria* was modelled as a normal distribution with mean = 23 Ma and standard deviation = 4, on the basis of an estimate obtained in a relaxed molecular-clock analysis of *ndh*F sequences of the tribe Antirrhineae (Appendix 1). The latter analysis incorporates a calibration of 74 Ma for the divergence time between Oleaceae and Antirrhineae (Bell *et al.*, 2010), and minimum stem-age constraints for Lamiales families and tribes based on five fossils (see Appendix 1 for details). Four MCMC analyses were run for 10 million generations, with a sample frequency of 1000. Parameter analysis in Tracer 1.4 (Rambaut & Drummond, 2007) showed adequate sample size, with effective sample size (ESS) values above 1000. Both chains were combined using LogCombiner 1.6.2, after discarding the first 10% of sampled generations as burn-in. Trees were summarized in a maximum clade credibility (MCC) tree obtained in TreeAnotator 1.6.2 and visualized in FigTree 1.3.1. Analyses performed using birth-death and Bayesian skyline tree priors yielded similar results. Therefore, only the birth-death chronogram is further discussed.

Genetic diversity and differentiation

In order to assess differences in cpDNA and *At103* genetic diversity across the geographic range of *L. elegans*, we calculated the number of haplotypes (*h*), number of private haplotypes (*ph*) and haplotypic diversity (*H*) (Nei, 1987) for each of the nine geographic areas (Fig. 2). The same parameters were also computed for the three major regions.

Geographic structure for the two loci was assessed using spatial analysis of molecular variance (SAMOVA; Dupanloup *et al.*, 2002). This analysis identifies groups of populations that are geographically homogeneous and maximally differentiated from each other. Analyses were repeated using values of *K* (number of groups) from 2 to 10, and all runs included 10,000 steps from each of 100 random initial conditions. The fitness of each configuration (value of *K*) was assessed by comparing values of F_{ct} (the proportion of total genetic variance resulting from differences among groups of populations). In addition, the nearest-neighbour statistic S_{nn} (Hudson, 2000) was calculated to assess genetic differentiation due to isolation among geographic areas. The datasets were divided into nine partitions corresponding to the nine geographic areas. The statistic was calculated using DnaSP, and permutation tests with 1000 replicates were performed to evaluate statistical significance.

Discrete phylogeographic analyses

In order to reconstruct the spread history of *L. elegans*, cpDNA and *At103* sequences were separately analyzed using the spatial diffusion methodology, a recently developed approach aimed to identify the ancestral geographical history of a sample of molecular sequences (Lemey *et al.*, 2009; Lemey *et al.*, 2010; Fernández-Mazuecos & Vargas, 2011a). Analyses were conducted in BEAST v.1.6.2 for the complete cpDNA and *At103* datasets, excluding outgroup sequences. We implemented a relaxed molecular clock, with an uncorrelated lognormal distribution for the substitution rate variation, and a Bayesian skyline was used as tree prior. Substitution models for the three cpDNA regions were unlinked (F81 for *rpl*32-*trn*L^{UAG}, GTR for *trn*K-*mat*K, and HKY for *trn*S-*trn*G, as inferred by jModelTest). The substitution model for *At103* was HKY+G. Based on previously estimated divergence times (see Results), the tree root height in both analyses was modelled as a normal distribution with mean = 1.17 Ma and standard deviation = 0.42.

In the cpDNA analysis, sequences yielding haplotype A were enforced as ancestral by placing a monophyly constraint affecting the remaining sequences, based on phylogenetic results (see Results). The phylogeographic history of both loci was reconstructed using the Bayesian phylogeographic framework described by Lemey *et al.* (2009). We defined eigth areas (AL, GP, CM, ES-GA, GR, GU, NI and SI), which were mapped through a discrete phylogeographic analysis that employs a standard continuous-time Markov chain (CTMC). As suggested by Lemey *et al.* (2009), we also implemented a Bayesian stochastic search variable selection (BSSVS) procedure to identify parsimonious descriptions of the diffusion process. Three MCMC analyses were run for 100 million generations, sampling every 10000th generation. Analysis with Tracer confirmed adequate sample sizes. The three chains were combined using LogCombiner 1.6.1 after discarding the first 10% of sampled generations as burn-in, and trees were summarized in a MCC tree obtained in TreeAnotator 1.6.1. Finally, a Bayes factor (BF) test was performed to identify rates (diffusion routes) that are frequently invoked to explain the diffusion process. Rates yielding a BF > 3 were considered as well supported (Lemey *et al.*, 2009).

Isolation with migration model

In the presence of the expected discordance between loci, coalescent-based statistical methods are needed for the reliable inference of phylogeographic patterns (Knowles & Maddison, 2002; Nielsen & Beaumont, 2009). Here we implemented a model of isolation with migration in IMa2 (Hey, 2010) in order to estimate split times, effective population sizes through time and migration rates between populations of the three major geographic regions (Cantabrian region, Central System and Iberian System). Split times are particularly relevant for the testing of our hypotheses (Fig. 1). The two extensively sampled sequence datasets (cpDNA and *At103*) were included as two independent loci. Based on area relationships suggested by DPA, we defined an input tree in which the Central System and Iberian System populations were sister to each other, and these were in turn sister to the Cantabrian region population (Supporting Information Fig. S1). This model was simple enough to maintain a reasonably small number of parameters to be estimated (15), at the same time allowing the testing of our hypotheses (Fig. 1). An infinite sites mutation model (Kimura, 1969) was implemented for both loci, and the inheritance scalar was set at 0.25 for cpDNA and 1 for *At103*. A range of mutation rates was calculated for each locus

($4.15x10^{-6}$ to $1.77x10^{-5}$ mutations per year for cpDNA; and $6.42x10^{-7}$ to $2.01x10^{-6}$ mutations per year for *At103*) based on the 95% highest posterior density intervals for the divergence time between *L. elegans* and outgroup sequences (*L. nigricans* for cpDNA and *L. clementei* for *At103*) estimated in the dating analysis in BEAST (see above). These ranges, together with a generation time of one year (given that *L. elegans* is an annual species) were included in the model in order to estimate parameters on demographic scales. Upper bounds for the prior distributions of population sizes, splitting times and migration rates were calculated on the basis of the largest geometric mean of the population mutation rates across populations, as suggested in the software documentation. We ran two analyses with twenty parallel Markov chains each. After 100,000 burn-in steps, a total of 20,000 trees were sampled in each run, with 100 steps between tree savings. A geometric heating model was applied, with parameters h1 = 0.96 and h2 = 0.9. Both analyses reached equilibrium after the burn-in period. Given that the two runs yielded similar results, they were combined for subsequent analyses. The final model was graphically summarized using IMfig (Hey, 2009).

Results

Species distribution models

The average species distribution model for current conditions (Fig. 3A) spanned the current species distribution, plus a few additional areas, such as high mountains of south-eastern Spain (Sierra Nevada) and narrow areas of the Pyrenees. The mean area under the receiver-operating characteristic curve (a measure of model fitness) for testing data was high (0.951), which supported the predictive power of the model. Standard deviation of the ten replicates was low. According to jackknife tests and response curves, two temperature (bio6 and bio5) and one precipitation (bio14) variables, followed by the acidic/basic variable, were shown to be the most informative variables for the model (results not shown).

The projection to the LIG (Fig. 3B) yielded a similar overall distribution, but with unequal occupation areas. While a wider distribution than the current species range was inferred in the Cantabrian region, suitability in the Central System and the Iberian System was restricted to narrow highland areas. A small area was again inferred in Sierra Nevada.

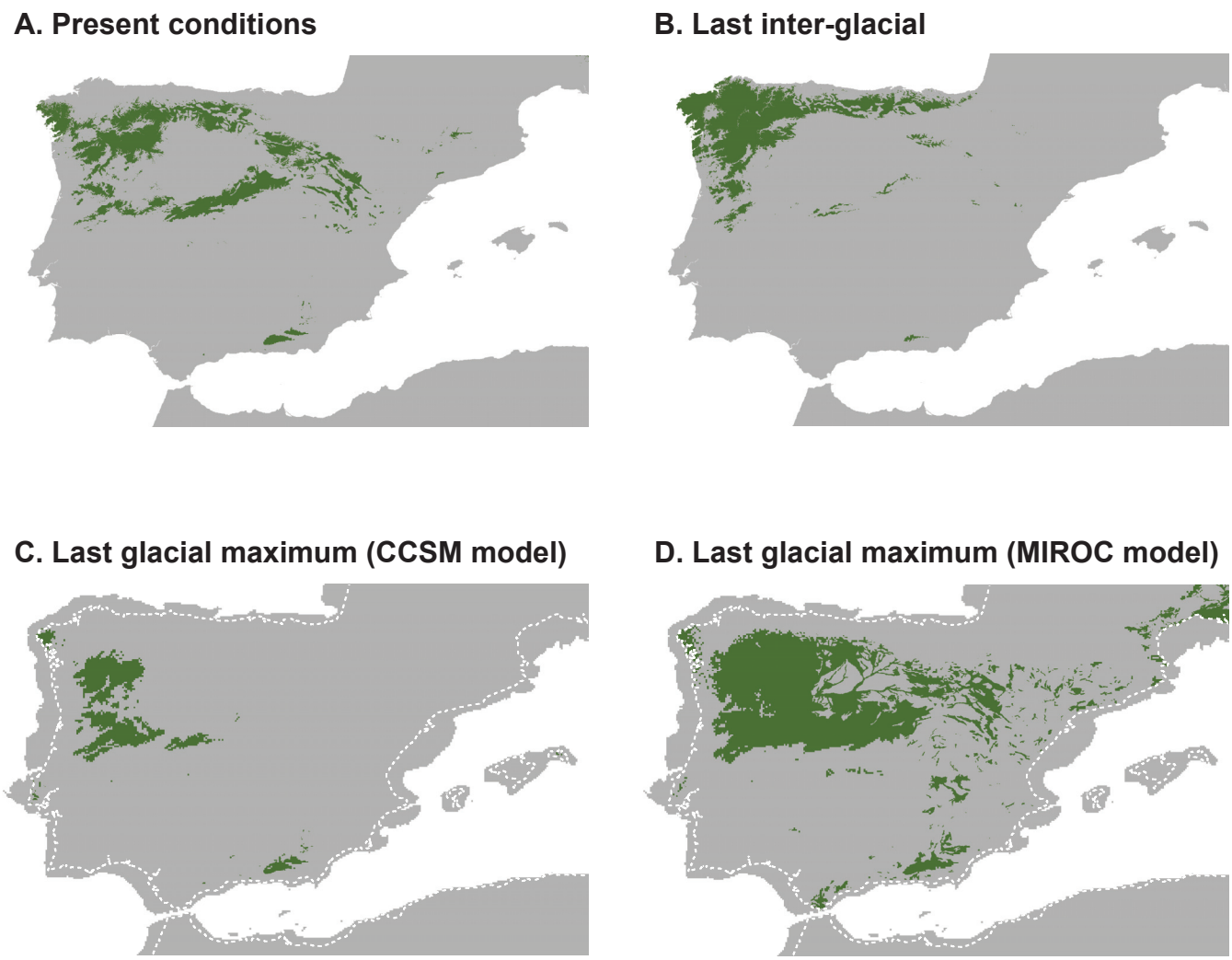


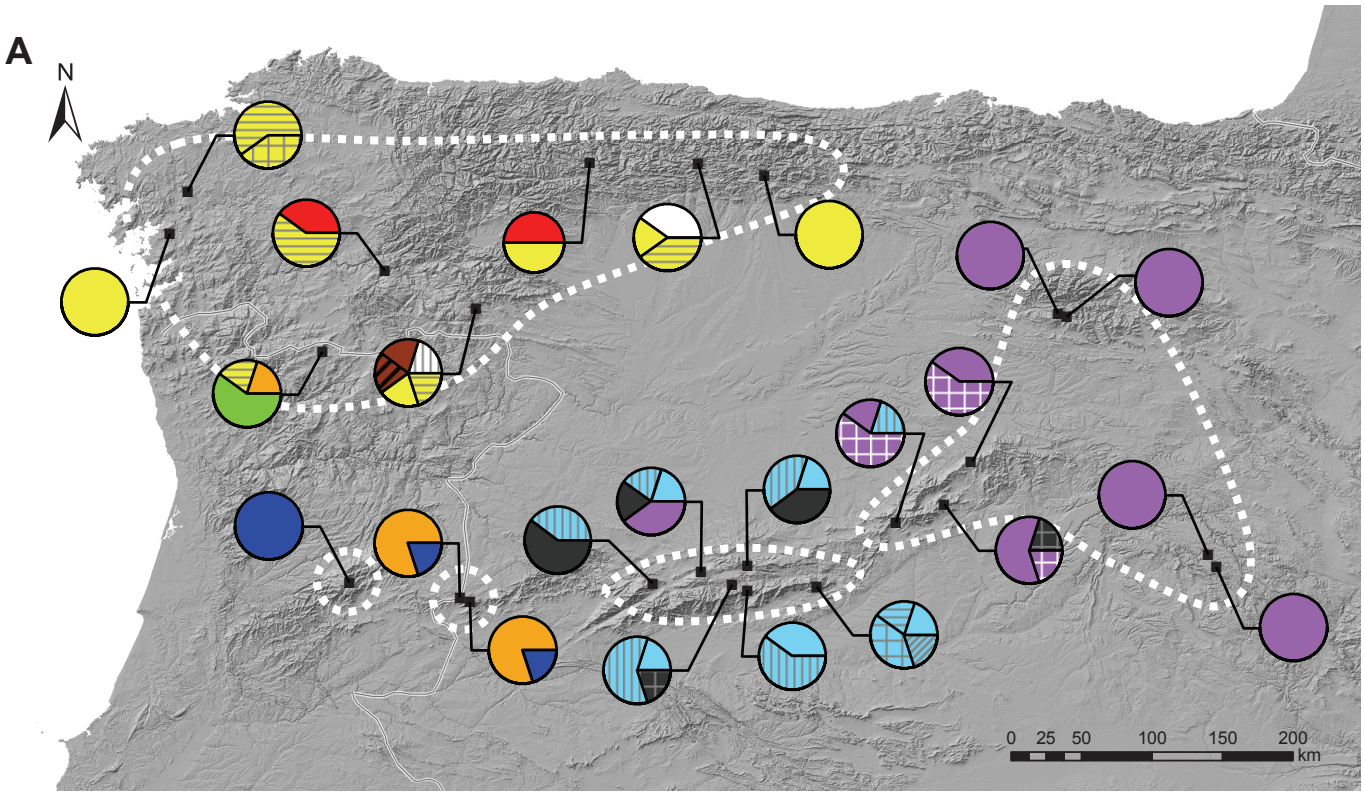
Fig. 3. Results of species distribution modelling of *Linaria elegans*. (A) Average distribution model fitted to current climatic conditions. (B) Average projection of the model to the last inter-glacial (c. 120-140 ka BP). (C) and (D) Average projections of the model to the last glacial maximum (c. 21 ka BP) using climatic variables under the CCSM (C) and MIROC (D) general circulation model simulations. LGM coastlines are represented in (C) and (D), with current coastlines superimposed as dotted lines.

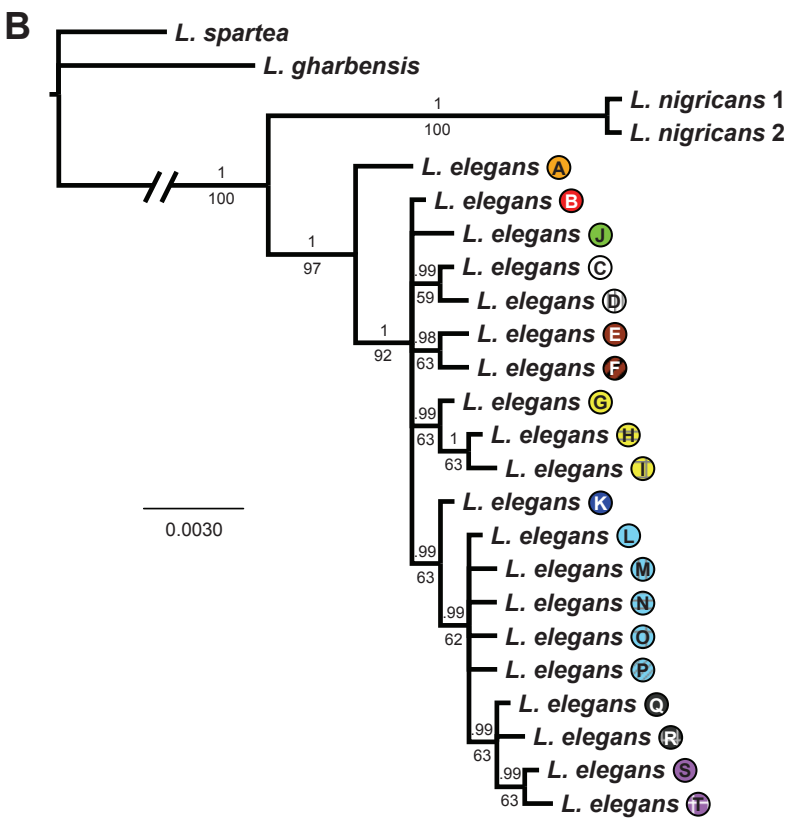
The CCSM and MIROC models for the LGM yielded strongly dissimilar inferences of *L. elegans* paleodistribution. The CCSM model (Fig. 3C) inferred a restricted potential distribution in north-western Iberia, including putative glacial refugia mostly in the western half of the Central System (ES, GA, GR and a small area in GU) and the western half of the Cantabrian region (GP, AL). Suitability was again inferred for Sierra Nevada. In contrast, the MIROC model (Fig. 3D) inferred a large distribution of suitable areas across the Iberian Peninsula, not only including the current distribution range, but also lower lands of the same regions, wide areas of the Duero basin and mountain ranges of south-eastern Spain.

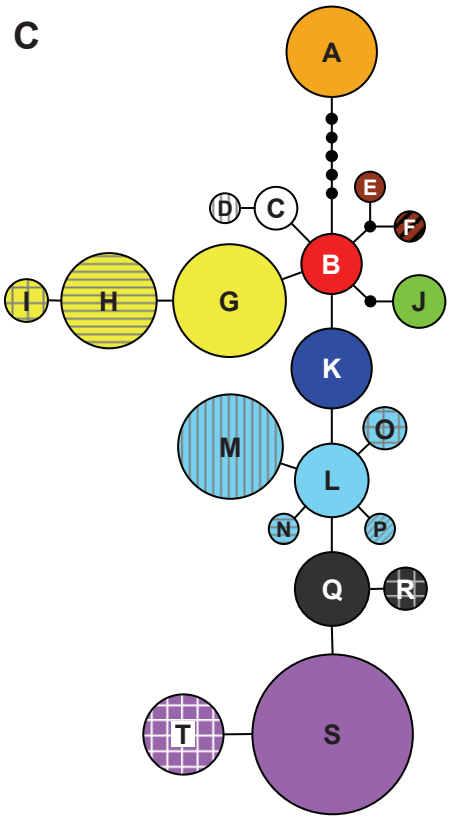
Analysis of cpDNA sequences

The combined analysis of the three cpDNA regions (2542 bp from 119 individuals; Figs. 4A-C) yielded 20 haplotypes of *L. elegans*, which formed a single network with no loops in the TCS analysis (Fig. 4C). Seven missing haplotypes were inferred, most of which (five) separated haplotype A from haplotype B. Haplotype B had the highest number of connections, specifically to six lineages formed by haplotypes A, C–D, E–F, G–I, J and K–T. Haplotypes and haplotype lineages showed distinct distribution ranges (Fig. 4A), with haplotypes B–J exclusively found in the Cantabrian region, the K–T lineage in the Central System and the Iberian System, and haplotype A in western segments of the Cantabrian region (GP) and the Central System (GA).

Fig. 4. Analysis of cpDNA (*rpl*32-*trn*L^{UAG}/*trn*K-*mat*K/*trn*S-*trn*G) haplotypes of *L. elegans*. The 20 cpDNA haplotypes are represented as fill patterns and colours, and named as in Table 2 and Supporting Information Fig. S4. (A) Geographical distribution of haplotypes across sampled populations. Pie charts represent haplotype proportions, obtained after sequencing 4-5 individuals per population. Population groups identified by SAMOVA for $K = 5$ are delimited by white dotted lines. (B) Fifty percent majority-rule consensus tree of the Bayesian phylogenetic analysis; numbers above branches are Bayesian posterior probabilities; numbers below branches are bootstrap supports (in percentage) of the maximum parsimony analysis. (C) Statistical parsimony network of cpDNA haplotypes; lines represent single nucleotide substitutions, and dots indicate missing haplotypes (extinct or not found). Circle sizes are proportional to the number of sequences obtained for each haplotype.


A
N
0 25 50 100 150 200
km


B
L. spartea
L. gharbensis
L. nigricans 1
L. nigricans 2
L. elegans A
L. elegans B
L. elegans J
L. elegans C
L. elegans D
L. elegans E
L. elegans F
L. elegans G
L. elegans H
L. elegans I
L. elegans K
L. elegans L
L. elegans M
L. elegans N
L. elegans O
L. elegans P
L. elegans Q
L. elegans R
L. elegans S
L. elegans T
0.0030


C
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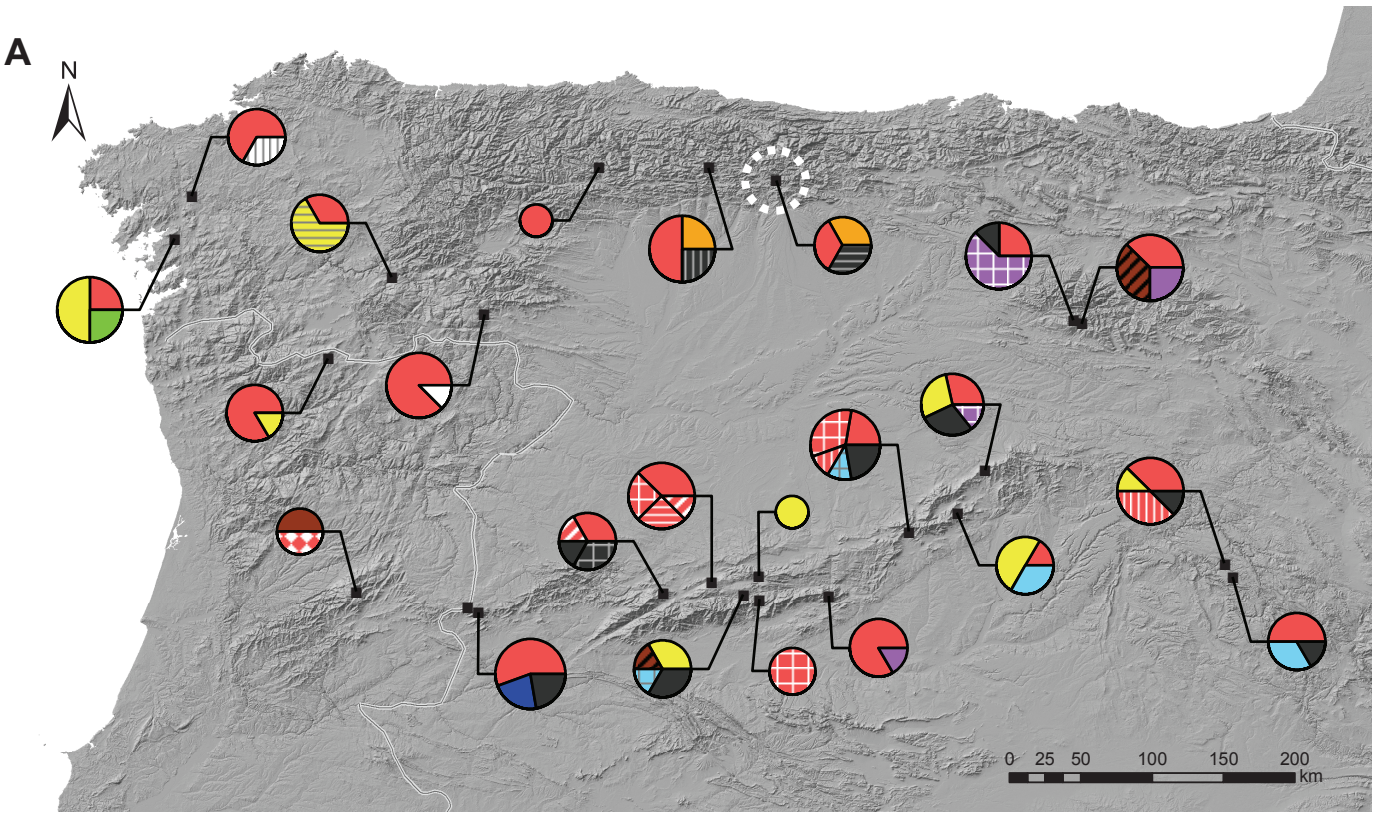
Seventeen populations yielded more than one haplotype, while the remaining seven were monomorphic populations, frequently found at peripheral locations (Table 2; Fig. 4A).

Relationships among haplotypes inferred by phylogenetic analyses (Fig. 4B) showed that *L. elegans* sequences formed a monophyletic group (posterior probability PP = 1; bootstrap support BS = 97%). Within the *L. elegans* clade, haplotype A was revealed as sister to a clade constituted by the remaining haplotypes (PP = 1; BS = 92%). Relationships within this clade were congruent with those inferred by the network analysis. Accordingly, haplogroups C–D, E–F, G–I and K–T were inferred as monophyletic, and haplotype K was inferred as the basal-most haplotype within the K–T clade.

Analysis of nuclear sequences

A total of 111 individuals provided unambiguous *At103* sequences. The aligned matrix had a total length of 391 bp. Haplotype reconstruction in PHASE resulted in highly supported haplotype pairs (probability > 0.90) for 81 individuals (162 sequences in total). The final recombination-free dataset obtained in IMgc had 147 *L. elegans* sequences from 75 individuals, and an aligned length of 230 bp. Analysis of this matrix in TCS yielded 23 haplotypes (Figs. 5A-C), 22 of which formed a network with no loops, while the remaining haplotype (1) was unconnected (Fig. 5C).

Fig. 5. Analysis of nuclear *At103* haplotypes of *L. elegans*. The recombination-free dataset obtained in IMgc was employed. The twenty-three *At103* haplotypes are represented as fill patterns and colours, and named as in Table 2. (A) Geographical distribution of haplotypes across sampled populations. Pie charts represent haplotype proportions, and pie chart sizes are proportional to the number of sequences obtained for each population. The single population separated from the remaining localities by SAMOVA (K = 2) is delimited by a white dotted line. (B) Fifty percent majority-rule consensus tree of the Bayesian phylogenetic analysis; numbers above branches are Bayesian posterior probabilities; numbers below branches are bootstrap supports (in percentage) from the maximum parsimony analysis. (C) Statistical parsimony network of *At103* haplotypes; lines represent single nucleotide substitutions, and dots indicate missing haplotypes (extinct or not found). Circle sizes are proportional to the number of sequences obtained for each haplotype.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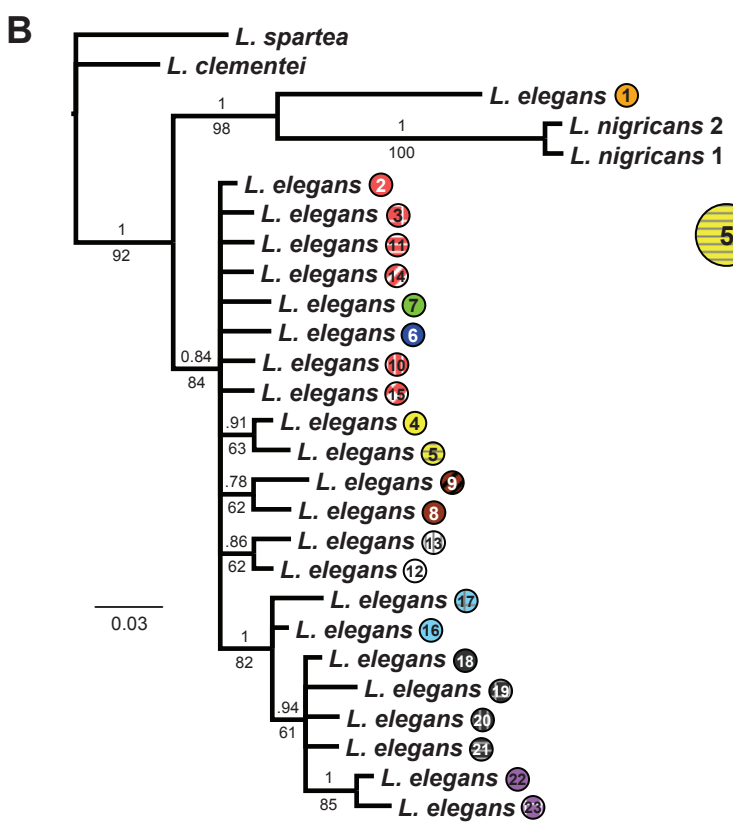
km


B
L. spartea
L. clementei
L. elegans 1
L. nigricans 2
L. nigricans 1
L. elegans 2
L. elegans 3
L. elegans 11
L. elegans 14
L. elegans 7
L. elegans 6
L. elegans 10
L. elegans 15
L. elegans 4
L. elegans 5
L. elegans 9
L. elegans 8
L. elegans 13
L. elegans 12
L. elegans 17
L. elegans 16
L. elegans 18
L. elegans 19
L. elegans 20
L. elegans 24
L. elegans 22
L. elegans 23
1
98
100
92
0.84
84
.91
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.78
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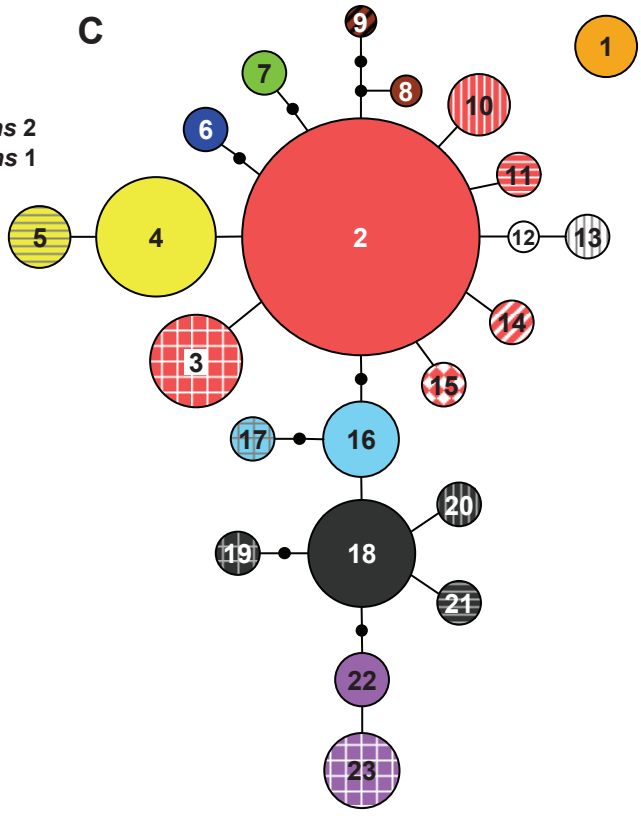
.86
62
82
.94
61
85
0.03


C
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Geographic structure (Fig. 5A) was poorer than that obtained from the cpDNA dataset. Some haplotypes were widely distributed (e.g. haplotypes 2, 4), while others were restricted to single or adjacent pairs of areas. Phylogenetic analyses (Fig. 5B) yielded relationships congruent with those of the network analysis. Monophyly of *L. elegans* sequences was retrieved (PP = 0.84; BS = 84%), except for haplotype 1, which was grouped with *L. nigricans*. We ascribed this pattern to incomplete sorting of ancestral polymorphisms (see Blanco-Pastor *et al.*, 2012), given the unlikely hybridization between *L. elegans* populations yielding haplotype 1 and *L. nigricans* (separated by c. 700 km).

In the phylogenetic analysis of the ITS region (595 bp), *L. elegans* sequences were grouped together as a monophyletic group (PP = 0.89; BS = 97%). They formed a large polytomy, except for a weakly-supported clade (PP = 0.7; BS < 50%) formed by sequences of western populations 5, 10 and 11 (Supporting Information Fig. S2). All populations (except for 9, 11 and 19) yielded a certain number (from 1 to 5) of additive polymorphic sites, detected as clear double peaks in electropherograms (Supporting Information Table S3). An examination of these sites in comparison with ITS sequences of the remaining species of *Linaria* sect. *Versicolores* (see Chapter 4) indicated that nucleotide additivities in *L. elegans* can be related to intraspecific variation, and not to interspecific hybridization. ITS sequences were not further used for haplotype analyses given their multicopy nature and concerted evolution (Álvarez & Wendel, 2003).

Genetic diversity and differentiation

Dissimilar patterns of genetic diversity and differentiation were encountered when analyzing plastid and nuclear *At103* sequences. For the cpDNA dataset, two geographic areas were found to harbour the highest genetic diversity (Table 3): GP in the Cantabrian region (h = 8; H = 0.867) and GR in the Central System (h = 8; H = 0.800). The same two areas had a high number of private haplotypes (four and five respectively). Three peripheral areas (ES, NI, SI) yielded no haplotypic diversity. When comparing the three major regions, the Cantabrian region and the Central System showed similar levels of genetic diversity, while the Iberian System showed no diversity (one haplotype). By contrast, *At103* diversity was more evenly distributed across geographic

areas, with values of haplotypic diversity ranging from 0.489 (GP) to 0.870 (GU) (Table 3). Private haplotypes were found in most areas, but not in GU, NI and SI. When considering the three major regions, the highest diversity and the highest number of private haplotypes were found in the Central System. Despite having the lowest haplotypic diversity, the Cantabrian region had a high number of private haplotypes (seven), while the Iberian System had none.

In the SAMOVA analysis of cpDNA sequences, high F_{ct} values were found for *K* values from 5 to 10 (Supporting Information Fig. S3A). For *K* = 5, all Cantabrian populations were included in a single group, while a strong geographic structure was found in the Central System (Fig. 4A), with four groups corresponding to the four geographic areas (ES, GA, GR, GU). Populations from the Iberian System were grouped with the Central System area GU. This basic structure was maintained for *K* values above 5 (Supporting Information Fig. S3C). When analyzing *At103* sequences, the highest F_{ct} was found for *K* = 2 (Supporting Information Fig. S3B). In this configuration, population 8 in the Cantabrian region was separated from all other populations (Fig. 5A). Values of the S_{nn} statistic (Supporting Information Table S4) were fully congruent with SAMOVA analyses.

Table 3. Genetic diversity parameters of population partitions of *Linaria elegans* using cpDNA and *At103* sequences (see Fig. 2 for geographic areas). Areas are sorted by cpDNA haplotypic diversity. *n* = number of sampled individuals; *h* = number of haplotypes; *ph* = number of private haplotypes; *H* = haplotypic diversity.

	cpDNA				***At103***			
	N	***h***	***ph***	***H***	***N***	***h***	***ph***	***H***
All populations	119	20	NA	0.900	147	23	NA	0.760
GP	15	8	4	0.867	20	4	2	0.489
GR	30	8	5	0.800	32	10	3	0.855
AL	10	3	1	0.689	14	4	2	0.747
CM	14	4	1	0.659	16	4	3	0.700
GU	15	4	1	0.657	22	8	0	0.870
GA	10	2	0	0.356	9	3	1	0.667
ES	5	1	0	0.000	4	2	2	0.667
NI	10	1	0	0.000	16	5	0	0.800
SI	10	1	0	0.000	14	5	0	0.780
Central System (ES, GA, GR, GU)	60	11	9	0.889	67	16	9	0.862
Cantabrian region (AL, GP, CM)	39	10	9	0.802	50	9	7	0.647
Iberian System (NI, SI)	20	1	0	0.000	30	8	0	0.825

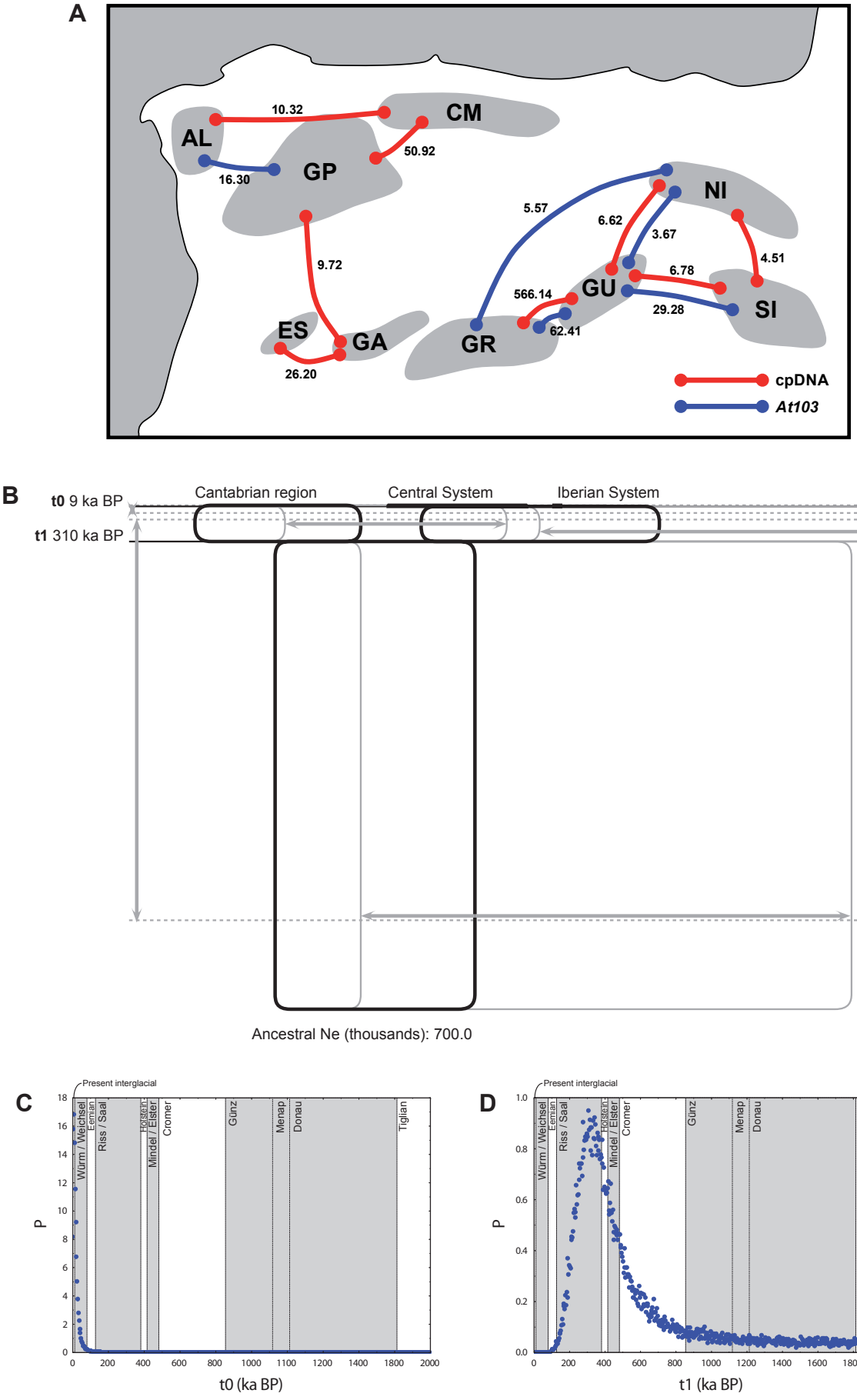
Divergence times of cpDNA lineages

The relaxed molecular-clock analysis (Supporting Information Fig. S4) estimated a divergence time between *L. nigricans* and *L. elegans* in the middle Pliocene to middle Pleistocene (0.89–3.79 Ma). Diversification of *L. elegans* haplotype lineages occurred during the Pleistocene, with haplotype A diverging at least 420 ka BP. The K–T lineage yielded a minimum stem-age of 270 ka, and a minimum crown-age of 140 ka.

Discrete phylogeographic analyses

The maximum clade credibility trees of the DPAs recovered a high uncertainty on the location of ancestors (results not shown). However, the BF analyses supported several different rates with BF values above 3 (Fig. 6A). Three connections were strongly supported by both cpDNA and *At103* sequences: GR-GU, GU-NI and GU-SI. The three areas within the Cantabrian region were connected by either cpDNA or nuclear sequences. The only supported connection between the Cantabrian region and the Central System was the western connection GP-GA supported by cpDNA. No connection was supported between the Iberian System and the Cantabrian region.

Fig. 6. Results of Bayesian phylogeographic analyses based on cpDNA and nuclear (*At103*) sequences of *L. elegans*. (A) Results of the discrete phylogeographic analyses (DPA). Geographic areas are delimited and named as in Fig. 2. Lines represent spread routes supported by Bayes factors (BF > 3) in DPA analyses of cpDNA (red) and nuclear *At103* (blue) sequences. Bayes factor values are also shown. (B) Summary of the isolation with migration model obtained in IMa2, based on the joint analysis of cpDNA and *At103* sequences. Each box represents a sampled or ancestral population, the height of which is proportional to the time it has lasted, and the width of which represents its effective size. Horizontal lines indicate splitting times. Ninety-five percent highest posterior density intervals for effective population sizes and splitting times are indicated (except for intervals referring to effective population sizes of current Central System and Iberian System populations, which have been omitted for clarity). (C) Marginal posterior probability distribution for splitting time t0 (Central System *vs.* Iberian System). (D) Marginal posterior probability distribution for splitting time t1 (Cantabrian region *vs.* Central System + Iberian System). European Quaternary stages are indicated in (C) and (D), with glacial periods in grey and inter-glacials in white (Silva *et al.*, 2009).


A
10.32
CM
AL
50.92
16.30
GP
9.72
NI
5.57
6.62
3.67
4.51
6.78
GU
566.14
29.28
SI
62.41
ES
GA
GR
26.20
cpDNA
At103
B
t0 9 ka BP
t1 310 ka BP
Cantabrian region
Central System
Iberian System
Ancestral Ne (thousands): 700.0
C
Present interglacial
Würm / Weichsel
Eemian
Riss / Saal
Holstein
Mindel / Elster
Cromer
Günz
Menap
Donau
Tiglian
P
t0 (ka BP)
D
t1 (ka BP)

Isolation with migration model

The isolation with migration model obtained in IMa2 (Figs. 6B-D; Table 4; Supporting Information Fig. S5) estimated an ancestral effective population size for the whole species around 700 thousands individuals. The split between the Cantabrian region population and the Central System-Iberian System populations (t1) was dated back to around 300 ka BP, and no less than 124.5 ka BP according to the 95% HPD interval (Figs. 6B, 6D; Table 4). By contrast, the split between the Central System and the Iberian System populations (t0) was estimated as a very recent event (around 9 ka BP, and no more than 63.1 ka BP according to the 95% HPD interval; Figs. 6B, 6C; Table 4). All migration rates were non-significant according to the test of Nielsen & Wakeley (2001).

Table 4. Estimated parameter values of the isolation with migration model obtained in IMa2 (Hey, 2012).

Parameter	Value with the highest probability	95% HPD interval
Divergence time t0 (Central System *vs.* Iberian System)	9 ka	0 – 63.1 ka
Divergence time t1 (Cantabrian region *vs.* Central System/Iberian System)	308.4 ka	124.5 ka – 3.6 Ma
Effective population size q0 (Cantabrian region)	589603 individuals	316785 – 1105929 individuals
Effective population size q1 (Central System)	497161 individuals	95825 – 1917620 individuals
Effective population size q2 (Iberian System)	25929 individuals	0 – 179248 individuals
Effective population size q3 (ancestral to Central System and Iberian System)	833111 individuals	422756 – 1732735 individuals
Effective population size q4 (ancestral to the whole species)	697829 individuals	296493 – 2023591 individuals

Discussion

Linaria elegans has long been recognized as a distinct taxonomic entity based on morphological characters (Viano, 1969; Sáez & Bernal, 2009). The observation of a well-supported monophyletic clade as based on the analysis of cpDNA sequences of *L. elegans* (Fig. 4B) corroborates these findings, and contrasts with the absence of monophyly obtained for several other species of *Linaria* sect. *Versicolores* (Fernández-Mazuecos & Vargas, 2011b; Chapter 3). This phylogenetic difference between *L. elegans* and other *Versicolores* species may have resulted from the old divergence of *L. elegans* from *L. nigricans* (0.89-3.79 Ma; Supporting Information Fig. S4), and the unlikely recent hybridization between these species given the geographical distance.

Modelling late Quaternary paleodistribution

The result of the SDM fitted to current conditions (Fig. 3A) closely resembled the current ring distribution of *L. elegans* (Fig. 2), except for certain peripheral areas, such as Sierra Nevada and the Pyrenees, where *L. elegans* is absent in spite of their environmental suitability. The latter mismatch derives from the fact that SDMs do not consider information on species history and assume species-climate equilibrium, namely that a species occupies all environmentally suitable areas (Nogués-Bravo, 2009). In the case of *L. elegans*, absence in these areas could be due to dispersal limitations (Svenning *et al.*, 2008). However, long-distance colonization across the Mediterranean region appears to have been relatively common in *Linaria* sect. *Versicolores* in the Quaternary, despite the absence of a long-distance dispersal syndrome (Fernández-Mazuecos & Vargas, 2011b; Chapter 3). Therefore, it is possible that the stochastic nature of colonization processes, rather than physical limitations, underlies the absence of *L. elegans* in Sierra Nevada and the Pyrenees.

The projection of the distribution model to the LIG also yielded a ring-shaped distribution (Fig. 3B), thus meeting the first assumption of the three hypotheses. However, the hypotheses were unequally supported by projections to the LGM (see diagrams in Fig. 1). Specifically, neither projection clearly supported Hypothesis II, which considered a lowland ring distribution during ice ages due to survival by altitudinal-descent migration to several isolated refugia.

The restricted distribution inferred under the CCSM model (Fig. 3C) included putative glacial refugia in western areas (AL, GP, ES, GA, GR), thus supporting Hypothesis III. On the other hand, the widespread distribution recovered by the MIROC model (Fig. 3D) included a fairly continuous range across the plateau, as postulated by Hypothesis I. Therefore, SDMs alone were not sufficient to determine which of the three proposed hypotheses constitutes the best description of the Quaternary history of *L. elegans*.

Phylogeographic hypothesis testing

The analysis of multi-locus data in a coalescent-based framework has the potential to provide insights into the demographic history of populations, including changes in population size, split times and migration rates (Heled & Drummond, 2008; Hey, 2010). This approach is particularly meaningful when different patterns from independent loci are found. In addition, the novel spatial diffusion approach to phylogeography (Lemey *et al.*, 2009; Lemey *et al.*, 2010) can provide information on rates and directions of spread. Both methodologies supply complementary results (Bloomquist *et al.*, 2010) and, together with species distribution modelling, allow to test our three hypotheses in a robust statistical framework.

Hypothesis I postulates admixture of *L. elegans* populations across the Duero basin during the LGM due to altitudinal-descent migration (as found in *Pinus sylvestris* by Robledo-Arnuncio *et al.*, 2005). Accordingly, it predicts a split between populations from different mountain ranges postdating the LGM (phylogeographic prediction 1; Fig. 1). This hypothesis can be rejected on the basis of our isolation with migration model, in which the major split across the basin (Cantabrian region *vs.* Central System/Iberian System) was estimated to have occurred between 124.5 ka BP and 3.6 Ma BP with 95% probability (Fig. 6D; Table 4). This interval does not overlap with, and is older than, the most recent estimates for the LGM (26.5 to 19–20 ka BP; Clark *et al.*, 2009) and the entire last glacial period (c. 10 to 114 ka BP; Gibbard & Van Kolfschoten, 2004). In fact, the peak of the posterior probability distribution is clearly placed around the Riss and Mindel glaciations (Fig. 6D). This old split is congruent with the strong signal of phylogeographic structure, as evidenced by the strong differentiation between northern and south-eastern areas, particularly for plastid markers (Fig. 4A; Supporting Information Table S4).

Hypothesis II postulates splits between populations from different mountain ranges older than the LGM. The estimated split across the Duero basin agrees with this prediction, but not with the split between Central System and Iberian System populations (Fig. 6C; Table 4). The latter split has been estimated to have occurred between present time and 63.1 ka BP with 95% probability, an interval that overlaps the last glacial period and includes the LGM. The value with the highest probability (9 ka) suggests that this split may have postdated the last glaciation (Fig. 6C). The linear spread pattern found between the Central System and the Iberian System (Fig. 6A) further indicate that Hypothesis II does not provide a good explanation for the Quaternary history of *L. elegans* (phylogeographic prediction 2; Fig. 1). In addition, under Hypothesis II we would expect a strong genetic structure due to relatively long-term isolation. When considering cpDNA markers, Cantabrian and Central System populations where clearly differentiated, and a strong structure was found in the Central System (Fig. 4A). However, no clear structure was found within the Cantabrian region, and Iberian System populations were clearly related to the eastern Central System populations (GU).

Hypothesis III, by contrast, found definite support in our phylogeographic results. The old divergence between northern and southern areas (Fig. 6D; Table 4) is consistent with the existence of at least two independent and isolated Quaternary refugia on both sides of the Duero basin. A post-glacial colonization of the Iberian System from the Central System is strongly suggested by the recent split between these regions and the small effective population size estimated in the Iberian System as compared to the Central System (Fig. 6B; Table 4), together with the spread routes between these areas statistically supported by plastid and nuclear DNA-based DPA (Fig. 6A). Fine-scale location of glacial refugia is suggested by the geographic distribution of genetic diversity and by the genetic structure revealed by SAMOVA analyses. We failed to find any clear structure within the Cantabrian region (Supporting Information Fig. S3), which suggests a single refugium. This may have been located in GP (where the highest cpDNA diversity and a high number of exclusive cpDNA haplotypes were found; Table 3), as also suggested by distribution modelling under the CCSM model (Fig. 3C). The strong geographic structure of cpDNA variation found in the Central System suggests at least four refugia (ES, GA, GR, GU) that have remained isolated to a certain degree. GR displayed the highest diversity. A spread route between GR and GU was strongly supported by both plastid and nuclear markers (Fig. 6A), which indicates that GU may have been colonized from GR at some point during the

Quaternary. On the other hand, the SAMOVA analysis suggested a colonization of the Iberian System from GU, as these regions constituted a single group in the cpDNA analysis (Fig. 4A). This is also supported by DPA based on plastid and nuclear DNA (Fig. 6A).

Although phylogeographic investigations on glacial refugia and post-glacial recolonization are usually restricted to the last cycle of climatic fluctuations (Hewitt, 2004), our coalescent-based model (Figs. 6B-D) suggests an older history of divergence between *L. elegans* populations. The estimated split times imply that the isolation between the northern and southern refugia dates back to the Riss or even the Mindel glaciation, and persisted throughout at least the Eemian interglacial and the Würm glaciation (Fig. 6D). The persistence of an old genetic structure throughout several Quaternary climatic cycles has been previously reported for a lizard species in this geographic context (Paulo *et al.*, 2001). In the case of *L. elegans*, the observed genetic divergence may have resulted from recurrent survival events in at least two isolated refugia (one north and one south of the Duero basin) during the Quaternary ice ages (Hewitt, 2004), followed by recolonization of the mountain ring during inter-glacial periods without admixture of the colonizing lineages. A similar pattern of genetic divergence between Cantabrian and Central System populations has been reported for *Saxifraga pentadactylis* (Vargas, 2003). Additional studies involving species with a similar ring distribution, such as *Cytisus oromediterraneus* (Cubas *et al.*, 2006), may enable the identification of consistent phylogeographic patterns for mountain plant taxa of the Iberian Peninsula.

Phylogeographic evidence supports paleoclimatic modelling

Incongruence between the two LGM projections of *L. elegans* paleodistribution probably derives from the different assumptions and methods of the CCSM and MIROC simulations, which generally result in a stronger decrease in temperature modelled by CCSM, as compared to MIROC (Alba-Sánchez *et al.*, 2010; Habel *et al.*, 2010). Indeed, the three most relevant variables for the *L. elegans* model display remarkable differences in the study area between the CCSM and MIROC simulation (particularly bio6, the minimum temperature of the coldest month; see Supporting Information Fig. S6). Significant differences between projections obtained under different paleoclimatic models are frequently found (Oláh-Hemmings *et al.*, 2010; Garcia-Porta

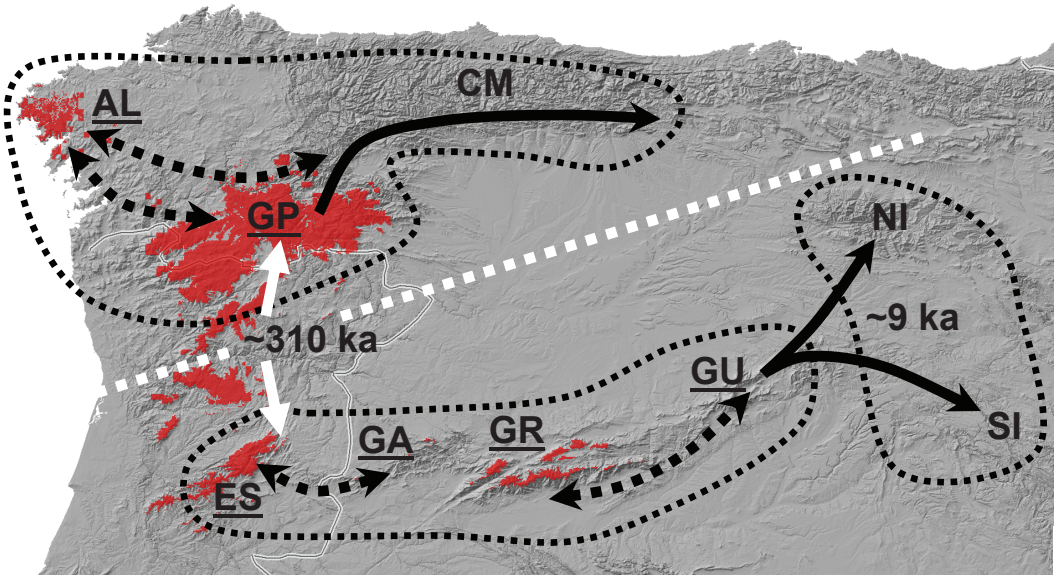


Fig. 7. Reconstruction of the late Quaternary history of *L. elegans* based on distribution modelling and phylogeographic analyses. Geographic areas are named as in Fig. 2. Major geographic regions are delimited by black dotted lines, and splitting times between them estimated by the isolation with migration model are indicated. The ancient split between north-western and south-eastern populations is represented by a white dotted line. Areas inferred as continuously suitable under LIG, LGM (CCSM model) and current climatic conditions (putative 'long-term refugia') are shaded in red. Areas suggested as refugia during the Last Glacial Maximum are underlined. Solid arrowed lines represent putative routes of post-glacial colonization. Dotted arrowed lines represent additional connections between areas.

et al., 2012; Rebelo *et al.*, 2012), and they are sometimes handled by calculating an average or consensus model (Waltari *et al.*, 2007; Abellán *et al.*, 2011; Flanders *et al.*, 2011). However, this approach does not seem appropriate in the presence of strong incongruence. Instead, independent biological data, such as those derived from phylogeographic or paleontological studies, are needed to validate the models.

Our integration of two sources of evidence (SDM and phylogeography) has enabled a reconstruction of the putative evolutionary and biogeographic history of *L. elegans* during the late Quaternary (Fig. 7). In this reconstruction, we have favoured the CCSM simulation of the LGM climate over the MIROC simulation. The CCSM-based model showed paleodistribution

patterns more consistent with DNA-based phylogeographic results. Had cycles of expansion across the northern Iberian plateau occurred during ice ages, as suggested by the MIROC model, the old split across the Duero basin, without subsequent significant migration (Fig. 6B), would not be expected. This latter phylogeographic pattern supports restricted glacial refugia, as inferred by the CCSM model.

The survival of populations in refugia is as important during warm periods as it is during cold periods to ensure the long-term persistence of a species (Bennett *et al.*, 1991). Five areas (GP, AL, ES, GA and GR) may be hypothesized as 'long-term refugia' (Stewart *et al.*, 2010) of *L. elegans*, as indicated by the observation that they harboured suitable habitats for the species throughout at least the last climatic cycle (Fig. 7). Additionally, GU may have harboured the species during the LGM (see the small area in Fig. 3C) after having been colonized from GR. From these refugia, two main routes of post-glacial recolonization can be suggested, one through the northern range (from GP to CM), and the other across central Iberia (from GU to the Iberian System), giving rise to the currently observed distribution. Admixture between both colonizing lineages in the north-east is not evidenced, as shown by the lack of significantly supported migration between the Cantabrian region and the Iberian System in the isolation with migration model. Such admixture would represent the next natural step to close the mountain ring, possibly giving rise to a suture-zone (Taberlet *et al.*, 1998), but it may have been hindered by the dominant calcareous substrates of this area.

The late Quaternary history of *L. elegans* does not fit classical models of glacial survival and post-glacial recolonization (Comes & Kadereit, 1998; Hewitt, 2000), inasmuch as a simple model of latitudinal shift towards warmer, southern regions during ice ages is not supported. The possibility of survival during this period through altitudinal shifts does not hold either. Instead, consistent refugia were found in western Iberian locations, which benefited from Atlantic climatic buffering. This suggests that the oceanic-continental gradient might have played a key and previously unrecognized role in determining the location of Quaternary refugia of certain species (Stewart *et al.*, 2010). Therefore, our results support the emerging complexity of Mediterranean peninsulas as glacial refugia (Gómez & Lunt, 2006; Schmitt, 2007; Médail & Diadema, 2009; Nieto-Feliner, 2011), as well as previous evidence for multiple colonization patterns of Mediterranean mountain plants (Vargas, 2003). They additionally provide a new

example of the historical complexity of ring distributions (Joseph *et al.*, 2008; Kuchta *et al.*, 2009; Mulcahy & Macey, 2009), a pattern that needs further investigation, particularly in the Mediterranean region.

Concluding remarks

The results showed that the integration of new methodologies for species distribution modelling and intraspecific phylogeography can enable faithful reconstructions of the late Quaternary history of plant species. This integrative approach is especially helpful as information from one source may help validate the patterns revealed by the other. This study used evolutionary evidence to favour the CCSM paleoclimatic reconstruction of the LGM over the MIROC reconstruction. There has been no investigation conducted to date that validates any of these models using independent biological data. Further research on additional taxa should help reduce the uncertainty of distribution models projected to the LGM by determining which model produces more consistent results.

Acknowledgements

We thank David Orgaz, Fidel Fernández-Mazuecos, Alberto Fernández-Mazuecos, Belén Estébanez, Alberto Bañón, Enrique Sánchez-Gullón, Bernardo García and José Luis Blanco-Pastor for field assistance; Jaime Güemes for plant material; Javier Amigo, Carlos Molina, José Luis Benito, Juan José Sánchez and Gonzalo Mateo for assistance in population finding; Emilio Cano, Gemma Andreu, Fátima Durán and Guillermo Sanjuanbenito for laboratory assistance; Jesús Muñoz, Juan Antonio Calleja and Beatriz Vigalondo for advice and comments on distribution modelling; José Luis Blanco-Pastor and Isabel Liberal for assistance with molecular analyses and insightful discussion. This research was supported by the Spanish Ministry of Science and Innovation through project CGL2009-10031, and by the Spanish Ministry of Education through a FPU fellowship (AP2007-01841) to the first author.

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Supporting Information

Fig. S1. Input tree of the isolation with migration analysis performed in IMa2 using two loci (cpDNA and *At103*).

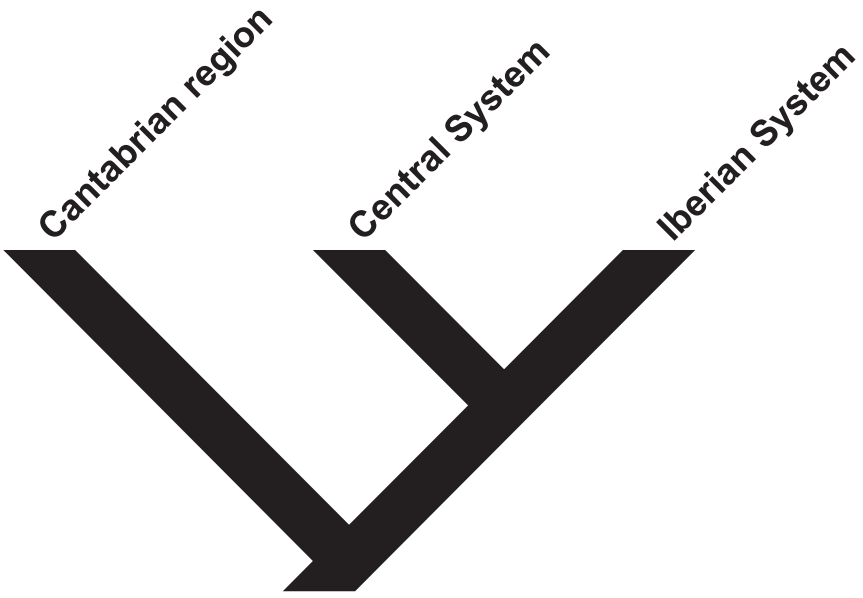

Fig. S2. Fifty percent majority-rule consensus tree of the Bayesian phylogenetic analysis of nrDNA ITS sequences. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are bootstrap supports (in percentage) from the maximum parsimony analysis. A hyphen (-) indicates no bootstrap support over 50%. Geographic areas and population numbers of sequenced individuals are indicated (see Fig. 2 and Table 1).

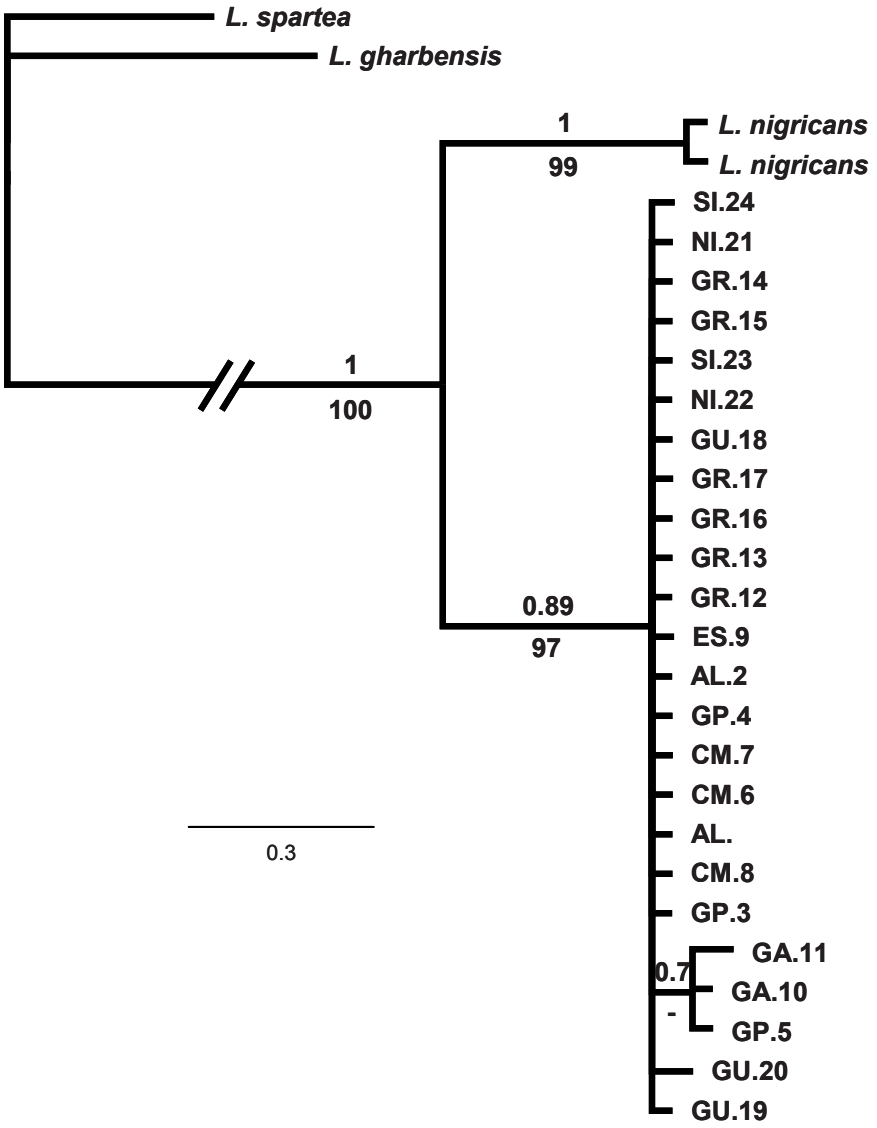

Fig. S3. Results of the spatial analyses of molecular variance (SAMOVA) in *L. elegans* populations. (A) F_{ct} values obtained when searching for K = 2 to 10 groups using cpDNA sequences. (B) F_{ct} values obtained when searching for K = 2 to 10 groups using nuclear *At103* sequences. (C) Population structure identified for cpDNA (red, K = 5 to 7) and At103 (blue, K = 2).

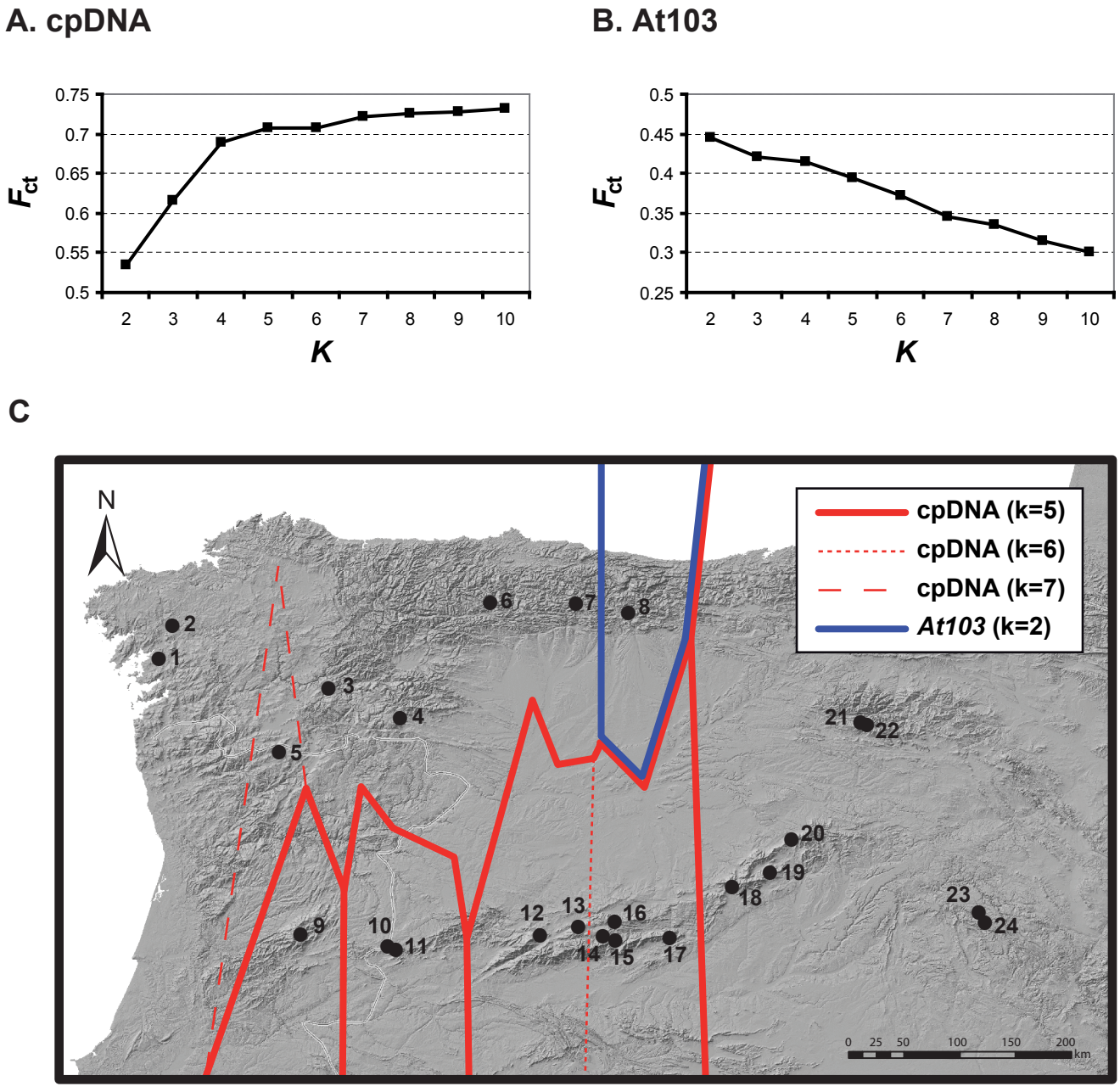

Fig. S4. Maximum clade credibility tree from the relaxed molecular-clock analysis of *rpl*32-*trn*L^{UAG}/*trn*K-*mat*K haplotypes. Only *L. nigricans* and haplotypes of *L. elegans* are shown. Node bars represent the 95% highest posterior density intervals for the divergence time estimates, which are also typed below branches. Numbers above branches are Bayesian posterior probabilities.

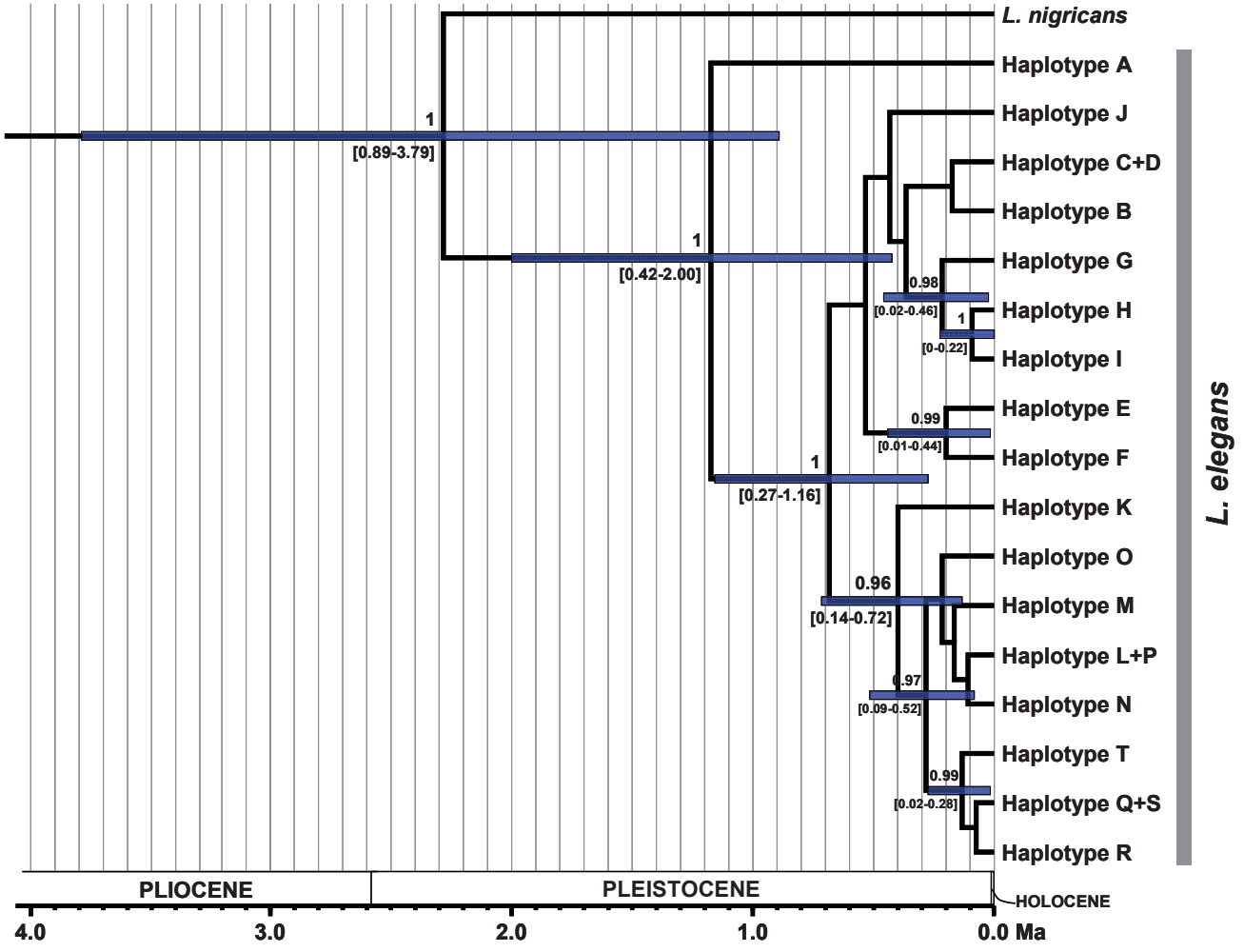

Fig. S5. Marginal posterior probability distributions for effective population sizes and migration rates of the isolation with migration model. Populations are labelled as follows. 0: Cantabrian region; 1: Central System; 2: Iberian System; 3: ancestral population to Central System and Iberian System; 4: ancestral population for the whole species. Direction of migration rates is interpreted in the coalescent direction, i.e. backwards in time. (A) Effective population size of population 0. (B) Effective population size of population 1. (C) Effective population size of population 2. (D) Effective population size of population 3. (E) Effective population size of population 4. (F) Population migration rate from 0 to 1. (G) Population migration rate from 0 to 2. (H) Population migration rate from 0 to 3. (I) Population migration rate from 1 to 0. (J) Population migration rate from 1 to 2. (K) Population migration rate from 2 to 0. (L) Population migration rate from 2 to 1. (M) Population migration rate from 3 to 0.

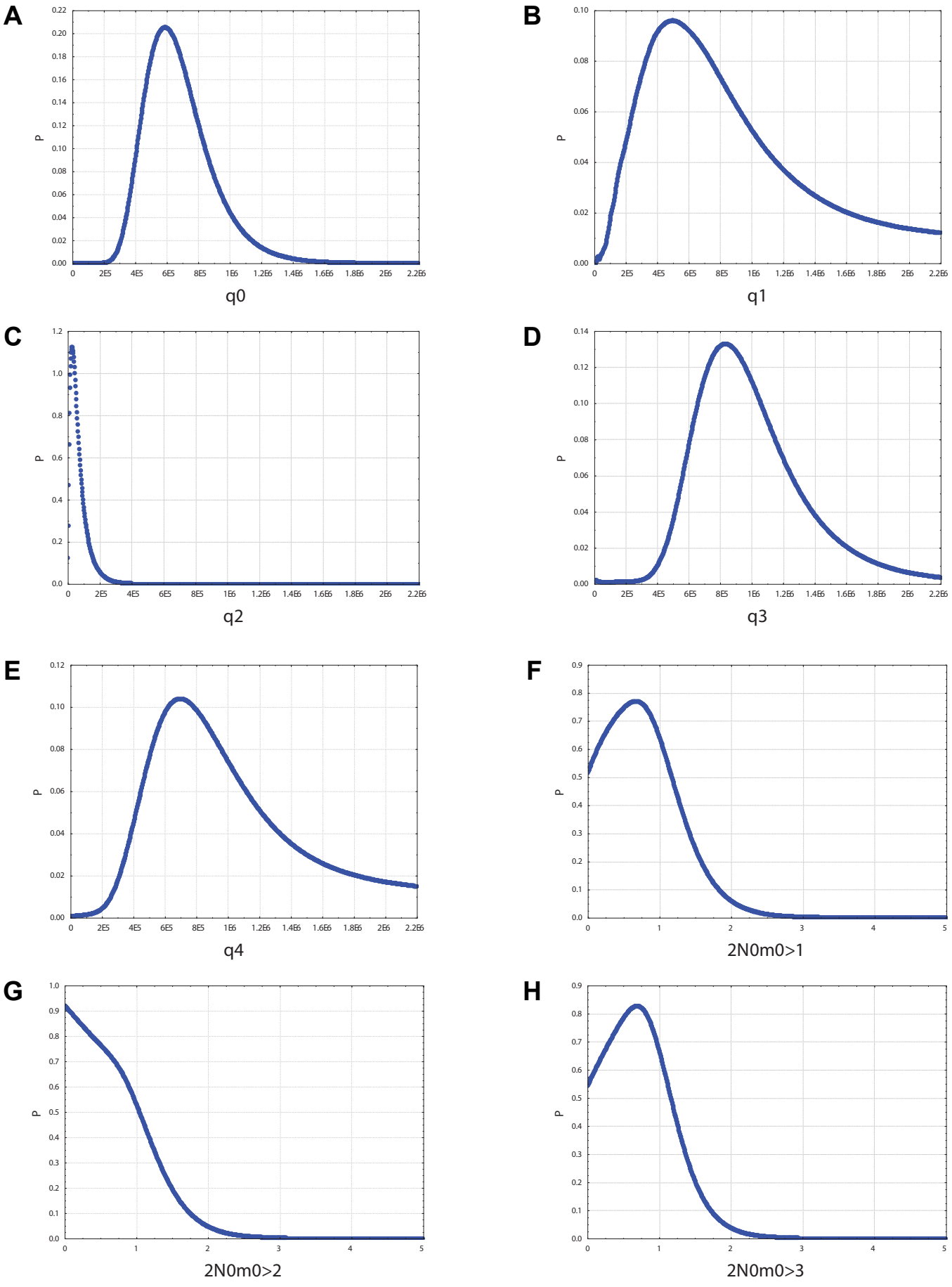
A
P
q0
B
P
q1
C
P
q2
D
P
q3
E
P
q4
F
P
2N0m0>1
G
P
2N0m0>2
H
P
2N0m0>3

Fig. S5. Continued.

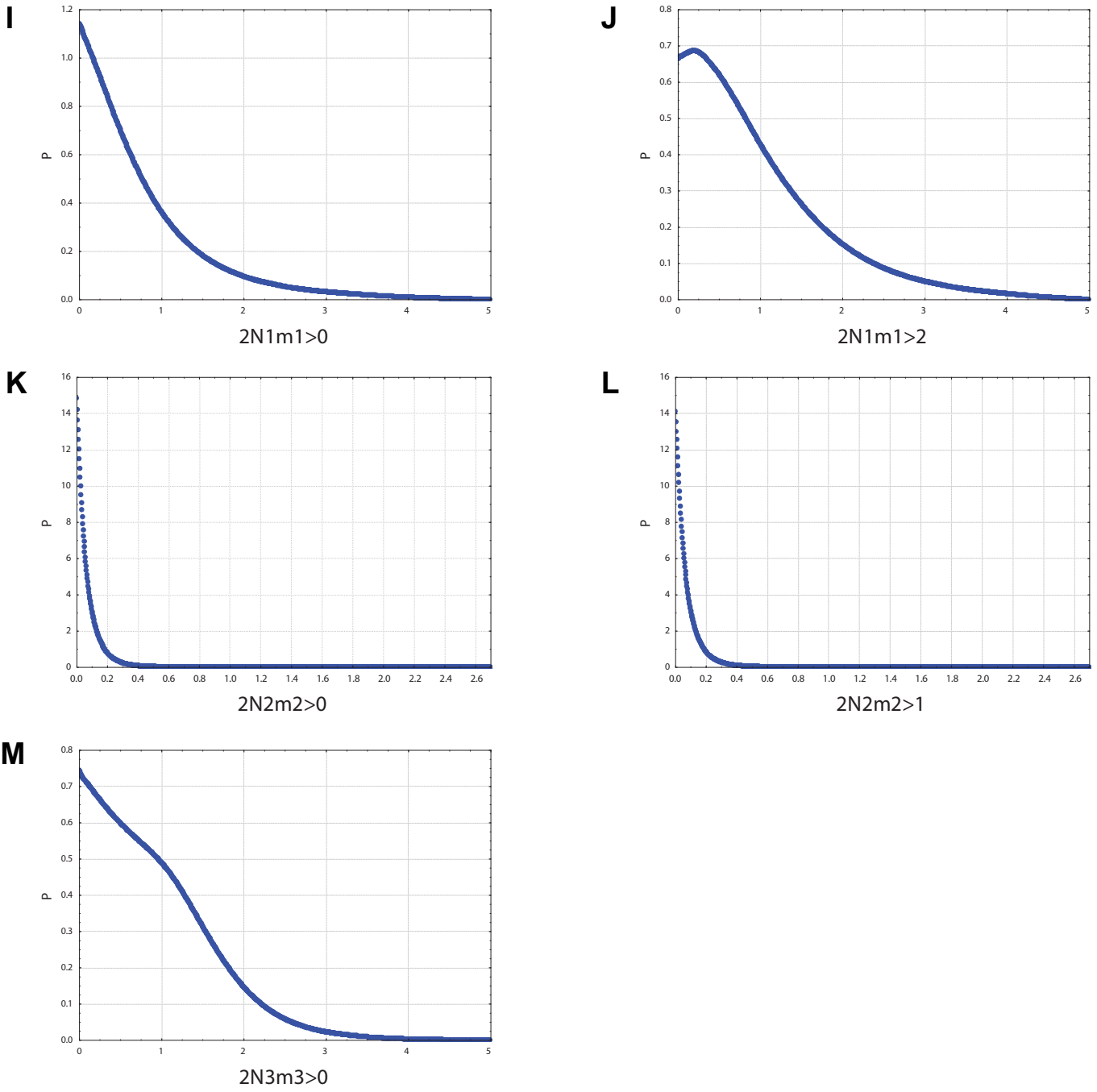

Fig. S6. Difference between LGM climate variables under two general circulation models (MIROC minus CCSM values) in the Iberian Peninsula. The three most informative variables of the Maxent distribution model for *L. elegans* are shown: (A) bio6 (minimum temperature of coldest month); (B) bio14 (precipitation of driest month); and (C) bio5 (maximum temperature of warmest month).

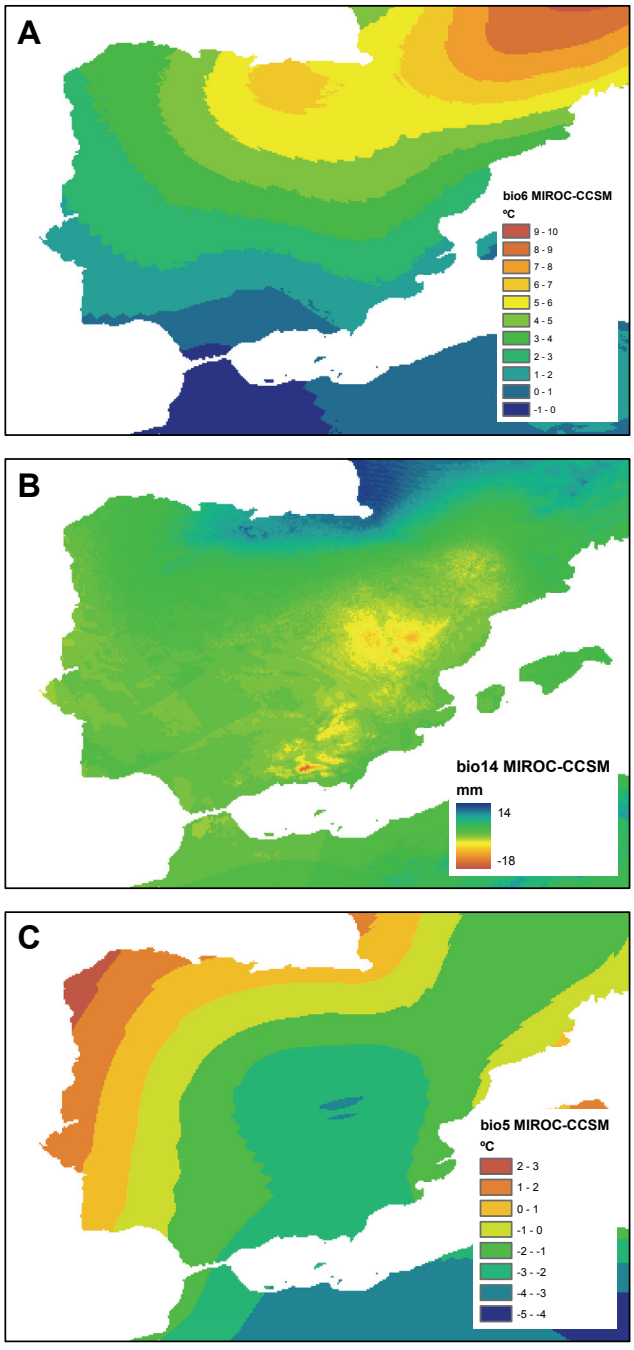

Table S1. Point localities of *L. elegans* employed in species distribution modelling.

Locality	Longitude	Latitude	Source
Portugal, Manteigas	-7.5502	40.3699	This chapter
Portugal, Mont Nave	-7.6810	40.9586	MA herbarium (Almeida, MA 791207)
Portugal, Montalegre	-7.7253	41.8414	This chapter
Portugal, Montalegre	-7.9211	41.8392	J. Güemes
Spain, A Coruña, Brión	-8.6941	42.8392	Camaño *et al.* (2005)
Spain, A Coruña, Rois	-8.7308	42.8303	Camaño *et al.* (2005)
Spain, A Coruña, Rois	-8.7554	42.8033	Pino Pérez *et al.* (2007)
Spain, A Coruña, Santiago de Compostela	-8.5849	42.8645	This chapter
Spain, Ávila, Becedas	-5.6393	40.3955	Sardinero Roscales (2004)
Spain, Ávila, Garganta de Bohoyo	-5.4363	40.3189	MA herbarium (P. Vargas, MA 756834)
Spain, Avila, Nava del Barco	-5.5649	40.2981	Sardinero Roscales (2004)
Spain, Ávila, Navalonguilla	-5.4918	40.2276	Sardinero Roscales (2004)
Spain, Ávila, Navarredonda de Gredos	-5.1122	40.3558	This chapter
Spain, Ávila, Puerto de Casillas	-4.5751	40.3433	This chapter
Spain, Ávila, Puerto de Menga	-5.0154	40.4732	This chapter
Spain, Ávila, Puerto de Mijares	-4.8054	40.3310	MA herbarium (A. Quintanar *et al.*, MA 806222)
Spain, Ávila, Puerto de Peñanegra	-5.3107	40.4357	This chapter
Spain, Ávila, Puerto del Pico	-5.0132	40.3216	This chapter
Spain, Ávila, Puerto del Tremedal	-5.6175	40.3644	This chapter
Spain, Ávila, Zapardiel de la Ribera	-5.3311	40.3391	Sardinero Roscales (2004)
Spain, Burgos, Cerro Grañón	-3.0331	42.0826	This chapter
Spain, Burgos, Neila	-2.9779	42.0639	This chapter
Spain, Cáceres, Eljas	-6.7827	40.2449	This chapter
Spain, Cáceres, Gargüera	-5.9657	40.0453	Amor *et al.* (1993)
Spain, Cáceres, Hervás	-5.8792	40.2186	MA herbarium (Itinera Mediterranea, MA 718688)
Spain, Cáceres, Piornal	-5.8168	40.1301	Sardinero Roscales (2004)
Spain, Cáceres, Puerto de Tornavacas	-5.6697	40.2687	Sardinero Roscales (2004)
Spain, Cuenca, El Tobar	-2.0787	40.5474	This chapter
Spain, Cuenca, Santa María del Val	-2.0266	40.4685	This chapter
Spain, Guadalajara, Ciruelos	-2.2261	41.0094	MA herbarium (C. Aedo, MA 754027)
Spain, Guadalajara, Luzaga	-2.4293	40.9918	Morales del Molino (2009)
Spain, Guadalajara, Solana Alto Cabrera	-1.6011	40.8045	Montserrat Martí & Gómez García (1983)
Spain, La Rioja, Viniegra de Abajo	-2.8910	42.1731	Medrano Moreno (1987)
Spain, León, Isoba	-5.3295	43.0436	This chapter
Spain, León, La Guiana	-6.5803	42.4448	Nieto Feliner (1985)
Spain, León, Lago de la Baña	-6.7450	42.2590	Nieto Feliner (1985)
Spain, León, Puerto de Ventana	-6.0215	43.0480	This chapter
Spain, León, Truchillas	-6.4671	42.2352	Nieto Feliner (1985)
Spain, León, Villafrea de la Reina	-4.9070	42.9650	This chapter
Spain, Lugo, A Pobra do Brollón	-7.2790	42.6826	Camaño *et al.* (2005)
Spain, Lugo, Cervantes	-6.8590	42.8286	Silva Pando (1994)
Spain, Madrid, Cercedilla	-4.0680	40.7530	This chapter
Spain, Madrid, El Escorial	-4.2400	40.5694	MA herbarium (P. Vargas, MA 778110)
Spain, Madrid, Puerto de Canencia	-3.7634	40.8690	This chapter
Spain, Madrid, Somosierra	-3.5903	41.1410	This chapter
Spain, Ourense, A Mezquita	-6.9956	42.0025	Camaño *et al.* (2005)
Spain, Ourense, A Ponte	-6.8786	42.2526	Camaño Portela *et al.* (2008)
Spain, Ourense, Barco de Valdeorras	-7.0061	42.4350	Pino Pérez *et al.* (2007)
Spain, Ourense, Lobios	-8.0848	41.8272	Camaño *et al.* (2005)
Spain, Ourense, Montederramo	-7.4844	42.2622	Pino Pérez *et al.* (2010)
Spain, Ourense, Oimbra	-7.4807	41.9109	Pino Pérez *et al.* (2009)
Spain, Ourense, Peña Trevinca	-6.7810	42.2687	Pino Pérez *et al.* (2008)
Spain, Ourense, San Xoán de Río	-7.3274	42.3575	This chapter

Table S1. Continued.

Spain, Ourense, Viana do Bolo	-7.0856	42.2202	Camaño Portela *et al.* (2009)
Spain, Palencia, Arroyo de Cueva Rodrigo	-4.7635	42.8890	García González (1990)
Spain, Palencia, Arroyo de la Muela	-4.8361	42.8609	García González (1990)
Spain, Palencia, Camporredondo de Alba	-4.7513	42.8892	García González (1990)
Spain, Palencia, La Rasa	-4.7742	42.8348	García González (1990)
Spain, Pontevedra, Caldas de Reis	-8.6956	42.5941	This chapter
Spain, Salamanca, Navasfrías	-6.8471	40.2735	This chapter
Spain, Segovia, Aldealengua de Pedraza	-3.8089	41.0264	García Adá (1995)
Spain, Segovia, Collado Hermoso	-3.9278	41.0255	García Adá (1995)
Spain, Segovia, La Granja	-4.0089	40.8807	García Adá (1995)
Spain, Segovia, Navafría	-3.8566	41.0350	García Adá (1995)
Spain, Segovia, Prádena	-3.6433	41.1265	García Adá (1995)
Spain, Segovia, Tres Casas	-3.9503	40.9352	García Adá (1995)
Spain, Teruel, Orihuela-Bronchales	-1.6545	40.5079	JACA herbarium
Spain, Teruel, Orihuela del Tremedal	-1.6777	40.5262	JACA herbarium
Spain, Teruel, Puerto de Bronchales	-1.6543	40.5169	Ruiz de la Torre (1979)
Spain, Teruel, Ródenas	-1.5099	40.6412	JACA herbarium
Spain, Teruel, Rodenos de Albarracín	-1.4210	40.3968	Ruiz de la Torre (1979)
Spain, Toledo, Navamorcuende	-4.7376	40.1513	Mateo & Pajarón (2004)
Spain, Toledo, Real de San Vicente	-4.7259	40.1515	Mateo & Pajarón (2004)
Spain, Zamora, Ribadelago	-6.7466	42.1184	This chapter
Spain, Zaragoza, Calcena	-1.7146	41.6706	JACA herbarium
Spain, Zaragoza, Purujosa	-1.8109	41.6626	Uribe-Echebarría (2009)
Spain, Zaragoza, Tarazona	-1.8085	41.7887	JACA herbarium
Spain, Zaragoza, Trasmoz	-1.7965	41.7886	Uribe-Echebarría (2009)

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Table S2. Sampled populations of *L. elegans* and outgroup species, with voucher specimens and geographic locations.

Taxon	(Population number) Locality	Voucher	Coordinates	Altitude (m)
Linaria Mill.				
Linaria sect. *Versicolores* (Benth.) Wettst.				
Subsect. *Versicolores*				
L. clementei Haensel. ex Boiss.	Spain, Huelva, Alhaurín de la Torre	M. Fernández-Mazuecos *et al.* 7MF08 (MA)	-	-
L. gharbensis Batt. & Pit.	Spain, Huelva, Gibraleón	M. Fernández-Mazuecos *et al.* 7MF09 (MA)	-	-
L. pedunculata (L.) Chaz.	Spain, Huelva, Marismas del Odiel	M. Fernández-Mazuecos *et al.* 3MF09 (MA)	-	-
L. spartea (L.) Chaz.	Spain, Madrid, Colmenar	P. Vargas 101PV07 (MA)	-	-
Subsect. Elegantes (Viano) D.A.Sutton				
L. elegans Cav.	(1) Spain, Pontevedra, Caldas de Reis	M. Fernández-Mazuecos 43MF09 (MA)	42°35'38.70"N 8°41'44.30"W	47
L. elegans Cav.	(2) Spain, A Coruña, Santiago de Compostela	M. Fernández-Mazuecos 42MF09 (MA)	42°51'52.30"N 8°35'5.50"W	169
L. elegans Cav.	(3) Spain, Ourense, San Xoán de Río	M. Fernández-Mazuecos 45MF08 (MA)	42°21'27.14"N 7°19'38.71"W	912
L. elegans Cav.	(4) Spain, Zamora, Ribadelago	M. Fernández-Mazuecos 48MF08 (MA)	42°7'6.20"N 6°44'47.80"W	1012
L. elegans Cav.	(5) Portugal, Montalegre	J. Güemes AH4209 (VAL)	41°50'29.00"N 7°43'31.00"W	930
L. elegans Cav.	(6) Spain, León, Puerto de Ventana	M. Fernández-Mazuecos 41MF08 (MA)	43°2'52.76"N 6°1'17.40"W	1473
L. elegans Cav.	(7) Spain, León, Isoba	M. Fernández-Mazuecos & D. Orgaz 40MF08 (MA)	43°2'36.92"N 5°19'46.20"W	1489
L. elegans Cav.	(8) Spain, León, Villafrea de la Reina	M. Fernández-Mazuecos & D. Orgaz 39MF08 (MA)	42°57'54.07"N 4°54'25.24"W	1160
L. elegans Cav.	(9) Portugal, Manteigas	M. Fernández-Mazuecos 127MF10 (MA)	40°22'11.80"N 7°33'0.89"W	1046
L. elegans Cav.	(10) Spain, Salamanca, Navasfrías	M. Fernández-Mazuecos 57MF09 (MA)	40°16'24.50"N 6°50'49.50"W	1014
L. elegans Cav.	(11) Spain, Cáceres, Eljas	M. Fernández-Mazuecos 58MF09 (MA)	40°14'41.50"N 6°46'57.70"W	1062
L. elegans Cav.	(12) Spain, Ávila, Puerto del Tremedal	M. Fernández-Mazuecos 33MF08 (MA)	40°21'51.66"N 5°37'3.07"W	1640
L. elegans Cav.	(13) Spain, Ávila, Puerto de Peñanegra	M. Fernández-Mazuecos & P. Vargas 41MF09 (MA)	40°26'8.50"N 5°18'38.70"W	1446
L. elegans Cav.	(14) Spain, Ávila, Navarredonda de Gredos	M. Fernández-Mazuecos 29MF08 (MA)	40°21'20.74"N 5°6'44.00"W	1605
L. elegans Cav.	(15) Spain, Ávila, Puerto del Pico	M. Fernández-Mazuecos 27MF08 (MA)	40°19'17.73"N 5°0'47.61"W	1385
L. elegans Cav.	(16) Spain, Ávila, Puerto de Menga	M. Fernández-Mazuecos 28MF08 (MA)	40°28'23.68"N 5°0'55.58"W	1555
L. elegans Cav.	(17) Spain, Ávila, Puerto de Casillas	M. Fernández-Mazuecos & B. Estébanez 31MF08 (MA)	40°20'35.74"N 4°34'30.40"W	1461
L. elegans Cav.	(18) Spain, Madrid, Cercedilla	M. Fernández-Mazuecos 26MF08 (MA)	40°45'10.91"N 4°4'4.69"W	1293
L. elegans Cav.	(19) Spain, Madrid, Puerto de Canencia	M. Fernández-Mazuecos 30MF08 (MA)	40°52'8.47"N 3°45'48.28"W	1527
L. elegans Cav.	(20) Spain, Madrid, Somosierra	M. Fernández-Mazuecos & A. Bañón 32MF08 (MA)	41° 8'27.64"N 3°35'25.08"W	1538
L. elegans Cav.	(21) Spain, Burgos, Cerro Grañón	M. Fernández-Mazuecos & B. Estébanez 73MF09 (MA)	42° 4'57.50"N 3°1'59.10"W	1688
L. elegans Cav.	(22) Spain, Burgos, Neila	M. Fernández-Mazuecos & B. Estébanez 72MF09 (MA)	42° 3'49.90"N 2°58'40.30"W	1236
L. elegans Cav.	(23) Spain, Cuenca, El Tobar	M. Fernández-Mazuecos 55MF09 (MA)	40°32'50.80"N 2°4'43.40"W	1304
L. elegans Cav.	(24) Spain, Santa María del Val	M. Fernández-Mazuecos 56MF09 (MA)	40°28'6.50"N 2°1'35.80"W	1364
L. nigricans Lange	Spain, Almería, Tabernas	P. Vargas 3PV08 (MA)	-	-
L. nigricans Lange	Spain, Almería, Cabo de Gata	M. Fernández-Mazuecos 29MF09 (MA)	-	-

Table S3. Thirteen variable sites of the nuclear ribosomal ITS region, sequenced for one individual per sampled population (see Fig. 2 for geographic areas). Polymorphic sites are shown as IUPAC symbols (K: G/T; M: A/C; R: A/G; S: C/G; W: A/T; Y: C/T; H: A/C/T).

		ITS sequence position												
Area	**Population**	**68**	**69**	**71**	**92**	**161**	**165**	**172**	**204**	**211**	**416**	**458**	**474**	**495**
-	*L. nigricans*	G	G	G	G	C	G	-	T	G	T	-	C	C
AL	1	A	A	A	G	C	G	C	Y	G	T	C	C	C
	2	A	A	A	G	C	G	C	T	G	T	M	C	C
GP	3	A	A	A	G	Y	S	C	T	G	T	C	C	C
	4	A	W	A	G	C	G	C	Y	G	T	M	C	C
	5	A	A	A	G	C	G	C	C	G	T	A	C	M
CM	6	A	W	A	G	C	G	C	Y	G	T	C	C	C
	7	A	W	R	G	Y	S	C	Y	G	T	C	C	C
	8	W	W	R	G	Y	S	C	T	G	T	C	C	C
ES	9	A	A	A	G	C	G	C	T	G	T	A	C	C
GA	10	A	A	A	G	C	G	C	C	G	T	A	M	C
	11	A	A	A	G	C	G	T	C	G	T	A	C	C
GR	12	A	A	A	K	C	G	C	T	G	T	M	C	C
	13	A	A	A	K	C	G	C	T	G	T	H	C	C
	14	W	W	R	K	C	G	C	T	G	T	H	C	C
	15	W	T	G	T	C	G	C	T	G	T	Y	C	C
	16	A	W	A	K	C	G	C	Y	G	T	H	C	C
	17	W	W	R	K	C	G	C	T	G	T	T	C	C
GU	18	T	T	C	T	C	G	C	T	G	W	Y	C	C
	19	T	T	G	T	C	G	C	T	G	T	T	C	C
	20	W	W	R	G	C	K	C	T	G	A	M	C	C
NI	21	W	W	R	K	C	G	C	T	G	T	C	C	C
	22	T	T	G	T	C	G	C	T	G	T	Y	C	C
SI	23	T	T	G	T	C	G	C	T	R	T	W	C	C
	24	W	W	R	T	C	G	C	T	G	T	C	C	C

Table S4. Nearest-neighbour statistic (S_{nn}) values calculated partitioning sequence datasets in order to evaluate genetic differentiation associated with isolation among areas (see Fig. 2 for geographic areas). Values for cpDNA are shown below the diagonal, and values for *At103* are shown above the diagonal. The statistic could not be calculated for the NI-SI comparison of the cpDNA dataset due to the lack of polymorphism. Abbreviations and symbols: NA, not applicable; ns, not significant; *, 0.01 < P < 0.05; **, 0.001 < P < 0.01; ***, P < 0.001.

	AL	**GP**	**CM**	**ES**	**GA**	**GR**	**GU**	**NI**	**SI**
AL	NA	0.670** (P=0.0010)	0.754** (P=0.0020)	1.000** (P=0.0040)	0.739* (P=0.0150)	0.719** (P=0.0030)	0.679** (P=0.0050)	0.800*** (P=0.0000)	0.695** (P=0.0070)
GP	0.749** (P=0.0020)	NA	0.689** (P=0.0010)	1.000*** (P=0.0000)	0.715** (P=0.0060)	0.708*** (P=0.0000)	0.743*** (P=0.0000)	0.771*** (P=0.0000)	0.672** (P=0.0050)
CM	0.597 ns (P=0.0970)	0.645* (P=0.0200)	NA	1.000** (P=0.0050)	0.733** (P=0.0090)	0.761*** (P=0.0000)	0.762*** (P=0.0000)	0.760*** (P=0.0000)	0.735*** (P=0.0000)
ES	1.000*** (P=0.0000)	1.000*** (P=0.0000)	1.000*** (P=0.0000)	NA	1.000* (P=0.0110)	0.995*** (P=0.0000)	1.000** (P=0.0010)	1.000*** (P=0.0000)	1.000** (P=0.0050)
GA	1.000*** (P=0.0000)	0.920*** (P=0.0000)	1.000** (P=0.0000)	0.778* (P=0.0100)	NA	0.725 ns (P=0.0530)	0.691* (P=0.0320)	0.698* (P=0.0360)	0.603 ns (P=0.1280)
GR	1.000*** (P=0.0000)	1.000*** (P=0.0000)	1.000*** (P=0.0000)	1.000*** (P=0.0000)	1.000*** (P=0.0000)	NA	0.525 ns (P=0.3650)	0.721** (P=0.0020)	0.679* (P=0.0200)
GU	1.000*** (P=0.0000)	0.993*** (P=0.000)	0.992*** (P=0.000)	0.950*** (P=0.0000)	0.960*** (P=0.000)	0.835*** (P=0.000)	NA	0.735*** (P=0.000)	0.536 ns (P=0.2810)
NI	1.000*** (P=0.0000)	1.000*** (P=0.0000)	1.000*** (P=0.0000)	1.000*** (P=0.0000)	1.000*** (P=0.0000)	0.909*** (P=0.0000)	0.631** (P=0.0070)	NA	0.718** (P=0.0040)
SI	1.000*** (P=0.0000)	1.000*** (P=0.0000)	1.000*** (P=0.0000)	1.000*** (P=0.0000)	1.000*** (P=0.0000)	0.909*** (P=0.0000)	0.631** (P=0.0040)	NA	NA

Capítulo 6

Discusión general

En la presente memoria doctoral, la aplicación de diversos métodos (filogenéticos, filogeográficos, morfométricos, de análisis filogenético comparativo y modelización de la distribución de especies) ha permitido comprobar la hipótesis general planteada en la introducción, así como dar respuesta a un amplio abanico de hipótesis particulares sobre la sistemática y evolución del género *Linaria*, y en particular de las linarias bífidas (*Linaria* sect. *Versicolores*). Algunas de estas hipótesis ya fueron sugeridas por autores anteriores, mientras que otras se proponen sobre la base de investigaciones propias. Nuestros resultados permiten contextualizar la abundante información disponible acerca del género (resumida en la Introducción) en un marco filogenético y evolutivo. Además, sugieren líneas futuras de investigación que serán de interés para profundizar en la comprensión de la evolución del grupo. Antes de resumir y discutir los principales resultados de esta memoria, vamos a comentar algunos desafíos metodológicos que se han presentado en el desarrollo de esta investigación, cuyo tratamiento puede ser de interés para investigaciones futuras similares.

Aspectos metodológicos

Los campos de la filogenia, la filogeografía, los métodos comparativos filogenéticos y la modelización de la distribución de especies se encuentran actualmente en una fase de rápido avance metodológico. A lo largo de esta memoria hemos aplicado algunos de los métodos de más reciente desarrollo en los cuatro campos. Pese a ello, las herramientas disponibles tienen limitaciones, a las cuales se deben enfrentar la mayoría de los estudios de filogenia y evolución que se desarrollan actualmente. Vale la pena comentar aquí algunos problemas metodológicos relevantes con los que nos hemos enfrentado, y la manera en la que los hemos tratado con el fin de dar cumplimiento a nuestros objetivos. Apuntamos también los avances que son previsibles en los próximos años, y que probablemente permitirán la superación de muchas de estas limitaciones.

1. *Delimitación de especies y reconstrucciones biogeográficas*

Una parte importante de los estudios biogeográficos que actualmente se publican se basan en análisis filogenéticos en los cuales se ha muestreado un único individuo por especie del grupo estudiado (p.ej. Roquet *et al.*, 2009; Buerki *et al.*, 2011; Valente *et al.*, 2011). En estos casos se asume implícitamente que ese individuo es representativo de la distribución completa de la especie, y se le asigna esa distribución de cara a la reconstrucción de áreas ancestrales. La consecuencia es el posible enmascaramiento de situaciones en las que una supuesta especie ampliamente distribuida está formada, en realidad, por un complejo de "especies crípticas" (Bickford *et al.*, 2007) con unos caracteres morfológicos muy similares, pero distribuidas en diferentes áreas y con un origen filogenético distinto. Si se obtienen secuencias de una sola de esas especies crípticas y se asigna a ellas la distribución de la totalidad del complejo, se introducirá un sesgo en las reconstrucciones biogeográficas. Este problema queda ilustrado, en nuestro análisis biogeográfico de *Linaria* sect. *Versicolores*, por el caso del complejo *L. incarnata* (Capítulo 3). En los últimos tratamientos, se había considerado a *L. incarnata* como una especie ibero-norteafricana (Viano, 1969, 1978b; Sutton, 1988), aunque ya se habían reconocido diferencias morfológicas entre las poblaciones ibéricas y las norteafricanas (Sutton, 1988). Al analizar secuencias tanto de individuos ibéricos como de individuos norteafricanos, los primeros quedaron incluidos en un clado exclusivamente ibérico, mientras que los segundos se incluyeron en un clado principalmente norteafricano (Capítulo 3, Capítulo 4, Apéndice 3). El análisis de caracteres morfológicos confirmó la existencia de ciertas diferencias entre ambos grupos de poblaciones y, junto con los análisis filogenéticos, justificó el reconocimiento de las poblaciones norteafricanas como una especie diferente, que se ha denominado *L. mamorensis* (Apéndice 3). En el caso de haberse secuenciado sólo un individuo ibérico de *L. incarnata* y de haberse asumido un estrecho parentesco entre las poblaciones ibéricas y las norteafricanas, en los análisis biogeográficos se habría inferido un evento de dispersión equivocado desde la península Ibérica al norte de África. A la inversa, de haberse secuenciado sólo un individuo norteafricano, se habría inferido un evento de dispersión equivocado en el sentido contrario. Por tanto, para la obtención de reconstrucciones biogeográficas fiables son aconsejables una delimitación de especies robusta y un buen muestreo del área de distribución de las especies, en particular de las ampliamente distribuidas, con el fin de detectar posibles especies crípticas surgidas por evolución convergente en distintas áreas. No obstante, en presencia

de incertidumbre taxonómica, nuestra aproximación basada en haplotipos de secuencias del ADN plastidial es apropiada para la reconstrucción de patrones de colonización por semillas (Capítulo 3), siempre que la herencia del genoma plastidial sea por vía materna, como ocurre en *Linaria* (Corriveau & Coleman, 1988).

2. *Reconstrucción de estados ancestrales y diversificación dependiente de carácter*

Hasta recientemente, la evolución de los caracteres a lo largo de los árboles filogenéticos se ha reconstruido mediante la utilización de métodos de parsimonia, máxima verosimilitud e inferencia bayesiana (Cunningham *et al.*, 1998; Huelsenbeck *et al.*, 2003) que asumen la independencia entre los estados de carácter y las tasas de diversificación del clado en estudio. Sin embargo, se ha argumentado y demostrado repetidamente que esa asunción puede conducir a sesgos en las reconstrucciones (Maddison, 2006; Goldberg & Igić, 2008). Así, cuando hay una asimetría en la distribución de los estados de un carácter a lo largo de un clado, ésta puede ser debida a una asimetría en las tasas de cambio del carácter (mayor tasa de cambio del estado menos frecuente al más frecuente) o bien a diferencias en la tasa de diversificación (mayor diversificación en el estado más frecuente que en el menos frecuente) (Maddison, 2006). Recientemente se han desarrollado herramientas analíticas para el estudio de los efectos de los estados de carácter sobre las tasas de especiación y extinción, las cuales permiten distinguir estos efectos de la existencia de asimetrías en las tasas de cambio del carácter (Maddison *et al.*, 2007; FitzJohn *et al.*, 2009; FitzJohn, 2010; Goldberg *et al.*, 2011). También se están desarrollando métodos que tienen en cuenta este problema en la reconstrucción de estados ancestrales (Goldberg & Igić, 2008). Nosotros hemos aplicado estos nuevos métodos al análisis de la evolución de la forma floral en *Linaria* sect. *Versicolores* (Capítulo 4). En efecto, se ha encontrado un efecto de determinados caracteres en las tasas de especiación, y esto ha posibilitado una reconstrucción más fiable de la evolución de estos caracteres. Estos métodos se han utilizado aún muy raramente para estudiar la evolución floral, pero es previsible que en los próximos tiempos se generalice su uso para todo tipo de características (FitzJohn *et al.*, 2009; FitzJohn, 2010), incluidas también las biogeográficas (Goldberg *et al.*, 2011).

3. *Hibridación, filogenia y reconstrucción de estados ancestrales*

El problema de la incongruencia entre marcadores es recurrente en la inferencia filogenética (Maddison, 1997; Wendel & Doyle, 1998). Se han descrito numerosas causas de incongruencia, entre las cuales las dos más citadas en plantas son la hibridación y la repartición incompleta de linajes (*incomplete lineage sorting* en inglés), también denominada coalescencia profunda (p.ej. Maureira-Butler *et al.*, 2008; Joly *et al.*, 2009; Blanco-Pastor *et al.*, 2012). Se han desarrollado distintos métodos para la inferencia de relaciones filogenéticas que tienen en cuenta uno u otro proceso. Así, varios métodos permiten la construcción de redes filogenéticas, gráficos de topología reticulada que representan adecuadamente la evolución en casos en los que la incongruencia entre marcadores se debe a eventos de hibridación (Huson *et al.*, 2011; Morrison, 2011). Por otro lado, se han desarrollado varios métodos basados en la teoría de la coalescencia para la inferencia de relaciones filogenéticas entre especies (árboles de especies o *species trees*) en presencia de incongruencia entre árboles de genes (o *gene trees*) debida a la coalescencia profunda (Liu, 2008; Heled & Drummond, 2010; Knowles & Kubatko, 2010). Unos y otros métodos asumen que toda la incongruencia se debe a una u otra causa. La distinción entre diferentes causas de incongruencia ha sido el objeto de numerosos estudios, y ha llevado al desarrollo de un buen número de métodos (Maureira-Butler *et al.*, 2008; Joly *et al.*, 2009; Yu *et al.*, 2011). Sin embargo, aún no se ha desarrollado un método estándar universalmente aceptado y aplicable para la inferencia de relaciones filogenéticas en presencia de ambas causas de incongruencia. Por otra parte, los métodos disponibles actualmente para la reconstrucción de estados ancestrales y el análisis filogenético comparativo se pueden aplicar sólo a relaciones filogenéticas en forma de árbol, no a redes filogenéticas. Dado que el cumplimiento de nuestros objetivos y el contraste de nuestras hipótesis dependen de este tipo de análisis (véase Introducción), hemos debido asumir una topología filogenética en forma de árbol y, por tanto, la inexistencia de eventos de hibridación relevantes en el origen de las especies de *Linaria* sect. *Versicolores* (Capítulos 3 y 4). Partiendo de esta asunción necesaria, toda la incongruencia entre marcadores que se ha encontrado (Capítulo 4) sería debida a la coalescencia profunda. Para tener en cuenta este proceso, se ha aplicado un método de reconstrucción de árboles de especies basado en la teoría de coalescencia recientemente desarrollado (Heled & Drummond, 2010). Distintos estudios han demostrado que los métodos de este tipo son claramente superiores a la concatenación de regiones de ADN en presencia de incongruencias (Edwards, 2009; Leaché & Rannala, 2011).

Se conoce bien la debilidad de las barreras reproductivas entre especies de la subsect. *Versicolores* (Valdés, 1970b; Viano, 1978a), hecho que se relaciona con el origen muy reciente (Cuaternario) de las especies, tal y como se ha inferido en nuestros análisis (Capítulo 4). Es posible que la hibridación haya jugado un papel relevante en el origen de ciertos táxones, como se ha encontrado en la sect. *Supinae* (Blanco-Pastor *et al.*, 2012), y que ésa sea una de las causas de la escasa resolución obtenida para las relaciones entre especies a escala detallada, junto con la reciente especiación (Capítulo 4). Cuando hay incertidumbre en las relaciones filogenéticas, como en este caso, los análisis evolutivos deben tener en cuenta esa incertidumbre (Huelsenbeck *et al.*, 2000). La aproximación más común para ello es efectuar los análisis en una muestra de árboles alternativos de semejante probabilidad, como las muestras generadas por los análisis bayesianos (Huelsenbeck *et al.*, 2001). Así, todos los análisis de reconstrucción de estados ancestrales, correlaciones y diversificación dependiente de carácter del Capítulo 4 se han efectuado sobre una muestra representativa de árboles de especies de la distribución bayesiana obtenida. Lo mismo se ha hecho para las reconstrucciones biogeográficas del Capítulo 3. En un futuro cercano, es previsible que se desarrollen nuevos métodos que modelicen la evolución de los caracteres a lo largo de redes filogenéticas que incorporen tanto la posibilidad de hibridación como la de coalescencia profunda. Entretanto, con los métodos disponibles, nuestra aproximación basada en el análisis de una serie de árboles de especies de similar probabilidad parece ser la más adecuada.

4. *Teoría de coalescencia y filogeografía multilocus*

En su origen, la filogeografía se basó en el análisis, en un marco geográfico, de linajes intraespecíficos obtenidos mediante secuencias de los genomas organulares mitocondrial (principalmente en animales) y plastidial (en plantas) (Avise *et al.*, 1987; Avise, 2000). Entre las ventajas de este tipo de datos genéticos se encuentran la ausencia de recombinación y la herencia generalmente materna de estos genomas (Avise, 2009). Pese a estas virtudes obvias, en los últimos años se han puesto de manifiesto las desventajas de utilizar una única región de ADN como fuente de datos filogeográficos (Knowles & Maddison, 2002; Nielsen & Beaumont, 2009). De hecho, según la teoría de la coalescencia, es esperable la obtención de patrones discordantes para distintos marcadores, y sólo el análisis conjunto de varios *loci* permite una

adecuada reconstrucción de la historia filogeográfica de las poblaciones de una especie (Brito & Edwards, 2009). En los últimos años se han desarrollado diversos métodos para el análisis filogeográfico de secuencias multilocus en el marco de la teoría de la coalescencia (Beaumont *et al.*, 2002; Heled & Drummond, 2008; Hey, 2010). Nosotros hemos empleado una de estas aproximaciones (Hey, 2010) para el análisis de secuencias de dos *loci*, uno nuclear y otro plastidial, de *L. elegans* (Capítulo 5). Pese a la discordancia de los patrones encontrados para cada marcador por separado, el análisis conjunto de ambos ha permitido una reconstrucción más fiable de la historia evolutiva de la especie, así como el contraste de las hipótesis planteadas. Además de los avances analíticos, el avance de las técnicas de secuenciación, y especialmente la facilidad cada vez mayor para la obtención de múltiples secuencias nucleares distribuidas a lo largo de todo el genoma, permitirán un gran avance de la filogeografía en los próximos años (Brito & Edwards, 2009).

5. *Modelos de distribución divergentes bajo diferentes simulaciones climáticas*

Los modelos de distribución de especies o modelos de nicho ecológico (Elith & Leathwick, 2009) se están utilizando cada vez más como complemento de la filogeografía en estudios de reconstrucción de la historia de las poblaciones (Richards *et al.*, 2007; Waltari *et al.*, 2007; Chan *et al.*, 2011). Estos modelos permiten inferir la distribución potencial de cualquier especie en la actualidad y proyectarla a condiciones diferentes (pasadas o futuras) de las actuales. Un problema común es la obtención de inferencias incongruentes al utilizar datos climáticos procedentes de distintas simulaciones (Garcia-Porta *et al.*, 2012; Rebelo *et al.*, 2012). Es obvia la necesidad de refinar las simulaciones climáticas, pero además, nosotros proponemos que la filogeografía proporciona datos independientes capaces de apoyar o refutar modelos de distribución alternativos (Capítulo 5). Así, en nuestro estudio de la historia evolutiva de *L. elegans*, los datos filogeográficos han apoyado una de las dos simulaciones climáticas del último máximo glaciar más utilizadas en la actualidad (CCSM frente a MIROC). Se requerirá más investigación para determinar si esta simulación (CCSM; Collins *et al.*, 2006) es consistentemente más fiable.

Implicaciones sistemáticas

De los análisis presentados en esta memoria se deriva una serie de resultados relevantes para la sistemática de *Linaria*. Estos resultados deberán ser tenidos en cuenta en futuros tratamientos del género, aunque en algunos casos se requerirán análisis adicionales para poder presentar una nueva propuesta sistemática. Nuestros resultados, basados en secuencias tanto del genoma nuclear (Capítulo 2) como del plastidial (Apéndice 2), apoyan la inclusión en *Linaria* de las cuatro especies americanas incluidas en *Nuttallanthus* por Sutton (1988). No obstante, el hecho de que las especies americanas formen un grupo monofilético, junto con la presencia de unos caracteres morfológicos bien definidos, apoyan el reconocimiento de estas especies como una sección aparte (*Linaria* sect. *Lectoplectron* Pennell). Con este resultado se terminan de aclarar las relaciones de parentesco entre los linajes de Antirrhineae del Nuevo Mundo y del Viejo Mundo. Algunos autores propusieron que muchas de las especies de Antirrhineae del Nuevo Mundo son congenéricas con las del Viejo Mundo y, por tanto, asignables a géneros como *Antirrhinum*, *Asarina* y *Linaria* (Pennell, 1935, 1947; Thompson, 1988). Sutton (1988), sin embargo, optó por separar todas las especies americanas en géneros distintos a los paleárticos. La filogenia de la tribu Antirrhineae más completa hasta el momento (Capítulo 2) apoya una solución intermedia, en la que el único género anfiatlántico es *Linaria*, con unas 150 especies en el Paleártico y cuatro en América (*L. canadensis*, *L. texana*, *L. floridana* y *L. subandina*). El resto de especies de América, por el contrario, pertenecerían a géneros endémicos de esta región, que están filogenéticamente aislados de los del Viejo Mundo (véanse también Vargas *et al.*, 2004 y Apéndice 1).

Dentro del género *Linaria*, la clasificación en dos grandes grupos de semillas aladas y ápteras respectivamente (Viano, 1978c) es claramente rechazada por nuestros análisis (Capítulo 2, Apéndice 2), tal y como también sugieren determinados caracteres morfológicos (Valdés, 1970a; Sutton, 1988). Tiene un mayor apoyo filogenético y evolutivo la clasificación en secciones iniciada por Bentham (1846) y Wettstein (1895), y posteriormente impulsada por Valdés (1970a) y Sutton (1988), aunque nuestros resultados sugieren ciertas modificaciones. Además de la ya comentada sección *Lectoplectron*, las secciones *Macrocentrum*, *Pelisserianae* y *Versicolores* son también grupos naturales definidos por sinapomorfías morfológicas claras (Valdés, 1970a; Sutton, 1980, 1988). Para la sect. *Supinae* se han obtenido resultados dispares

al contrastar secuencias nucleares (Capítulo 2) y plastidiales (Apéndice 2). Recientes análisis basados en simulaciones del proceso de coalescencia sugieren que *Supinae* es también un grupo monofilético (Blanco-Pastor *et al.*, 2012) y que la incongruencia entre marcadores se debe tanto a la repartición incompleta de linajes (*incomplete lineage sorting*) como a eventos recientes de hibridación. La polifilia de la sect. *Diffusae* es apoyada tanto por las secuencias nucleares (Capítulo 2) y plastidiales (Apéndice 2), como por la disparidad morfológica que se puede encontrar en este grupo (Valdés, 1970a; Sutton, 1988). Nuestros análisis y los de Blanco-Pastor *et al.* (2012) sugieren su desintegración en al menos dos secciones: sect. *Diffusae s.s.* y sect. *Minutiflorae* (Bentham, 1846; Valdés, 1970a). No obstante, se requieren más análisis antes de llegar a una propuesta definitiva. También se requerirán análisis adicionales, similares a los de Blanco-Pastor *et al.* (2012), para aclarar el estatus de las secciones *Linaria* y *Speciosae*, dada la clara incongruencia encontrada entre los resultados basados en secuencias nucleares (Capítulo 2) y plastidiales (Apéndice 2). Pese a la diferencia en el tipo de semilla (alada en la sect. *Linaria* y áptera en la sect. *Speciosae*), el resto de caracteres morfológicos (Sutton, 1988) y la alta capacidad de hibridación (Valdés, 1970b; Viano, 1978a; Ward *et al.*, 2009) relacionan estrechamente a estas dos secciones, al igual que nuestro análisis basado en secuencias nucleares ITS (Capítulo 2) y, en parte, el basado en secuencias plastidiales (Apéndice 2).

La sección *Versicolores* se ha confirmado como uno de los linajes de *Linaria* mejor definidos, tanto desde el punto de vista morfológico (Sutton, 1988) como filogenético (Capítulos 2-4, Apéndice 2). Asimismo, nuestros análisis apoyan inequívocamente la sistemática infraseccional propuesta por Sutton (1988), con dos subsecciones, *Elegantes* y *Versicolores*, que corresponden a grupos monofiléticos bien apoyados y con una relación de grupo hermano. Estos dos grupos se distinguen por la forma del estigma y de la parte terminal del estilo: estigma emarginado en la subsect. *Elegantes* y estigma bífido, con ramas estilares discretas, en la subsect. *Versicolores* (Viano, 1978b, c; Sutton, 1988).

En cuanto a la delimitación de especies, se ha analizado en detalle la sistemática del complejo *L. incarnata* (Viano, 1969; Sutton, 1988), y se ha llegado a la conclusión de que está formado por tres especies muy similares desde el punto de vista morfológico (especies crípticas; Bickford *et al.*, 2007): *L. mamorensis* (que se describe como especie nueva para la ciencia), en Marruecos; *L.*

onubensis, en el suroeste de España (Pau, 1933); y *L. incarnata s.s.*, en Portugal y el centro-oeste de España (Apéndice 3).

La identidad del endemismo griego *L. hellenica* como especie independiente había sido puesta en duda por Tan & Iatrou (2001), que asimilaron este taxon a *L. tenuis*, una especie ampliamente distribuida por el noreste de África. Nuestros análisis (Capítulos 3 y 4) no relacionan a *L. hellenica* con los individuos secuenciados de *L. tenuis*, y por tanto, apoyan la independencia taxonómica de *L. hellenica*. Esta es, además, una especie tetraploide, lo que constituye un caso único en la sección *Versicolores* (2n = 24, 26; Contandriopoulos & Yannitsaros, 1975), mientras que *L. tenuis* es, hasta donde se sabe, una especie diploide (Díaz Lifante *et al.*, 1992). Nuestros resultados filogenéticos confirman que la tetraploidía de *L. hellenica* es el resultado de un evento reciente de poliploidización, como había sugerido Sutton (1988).

Por otra parte, los resultados filogenéticos del Capítulo 4 sugieren la necesidad de una revisión del estatus de las especies politípicas –formadas por varias subespecies– de la sect. *Versicolores*: *L. viscosa*, *L. multicaulis* y *L. bordiana*. La mayoría de las subespecies presentan caracteres morfológicos bien definidos (Sutton, 1988; Sáez & Bernal, 2009). En el caso de *L. bordiana*, las flores de las dos subespecies pertenecen incluso a tipos morfológicos distintos (Capítulo 3). En los análisis filogenéticos no se han obtenido grupos monofiléticos apoyados que agrupen a las distintas subespecies de una misma especie (Capítulos 3 y 4). Análisis más detallados permitirán esclarecer en qué casos se trata, en realidad, de especies distintas, tal y como se describieron originalmente la mayoría de estos táxones (véase Sutton, 1988).

Una visión integradora de la evolución de *Linaria* sect. *Versicolores*

Los resultados de los Capítulos 3, 4 y 5 nos permiten proponer una reconstrucción de la historia evolutiva de *Linaria* sect. *Versicolores* en un marco espacial y temporal. Antes de ello, es necesario aclarar un punto. Los análisis de datación del Capítulo 3 y de los Capítulos 4 y 5 se han basado en calibraciones ligeramente distintas. En los Capítulos 4 y 5, las filogenias de la sect. *Versicolores* se calibraron con una edad basal de divergencia entre *Linaria* y *Chaenorhinum*

de 23 Ma, sobre la base de la estimación obtenida en la filogenia datada de la tribu Antirrhineae presentada en el Apéndice 1. Ésta se calibró, a su vez, utilizando una calibración secundaria basada en la filogenia datada más reciente de todas las angiospermas (Bell *et al.*, 2010), que se publicó durante el desarrollo de la presente memoria doctoral. En el Capítulo 3, por el contrario, se calibró la divergencia entre *Linaria* y *Chaenorhinum* con una edad de 29 Ma, sobre la base de una versión preliminar del análisis del Apéndice 1. En esta versión preliminar se utilizó una calibración secundaria basada en la filogenia datada del clado Asteridae de Bremer *et al.* (2004), la mejor disponible en el momento de la elaboración del Capítulo 3 (Fernández-Mazuecos & Vargas, 2011). Pese a la diferencia en la calibración, todos los análisis de datación de la sect. *Versicolores* dieron resultados esencialmente congruentes, con un ancestro común de la sección en el Mioceno Superior o el Plioceno, y una diversificación de especies principalmente en el Cuaternario (compárese la Fig. 2 del Capítulo 3 con la Fig. 4 del Capítulo 4).

Los distintos resultados filogenéticos y filogeográficos de esta memoria, por tanto, se pueden integrar de cara a obtener una visión general de la evolución de la sect. *Versicolores* en el espacio y en el tiempo. Esta reconstrucción permite confirmar nuestra hipótesis general relativa a una influencia determinante tanto de factores históricos abióticos como de factores bióticos en la evolución de *Linaria* sect. *Versicolores*. Dentro de los factores abióticos, se debe considerar el marco paleobiogeográfico en el que se ha desarrollado la evolución de las linarias bífidas. Desde finales del Mioceno, varios procesos históricos han tenido una influencia apreciable sobre la evolución y la biogeografía de las biotas, y en particular de la flora, en lo que hoy es la región Mediterránea (Thompson, 2005). A continuación se muestran los resultados más notables referentes a factores abióticos y bióticos que han influenciado la evolución de nuestro grupo de estudio:

1. *La historia de conexiones y barreras entre las placas Euroasiática y Africana*

Desde finales del Mesozoico, la progresiva aproximación de estas dos placas dio lugar al cierre del antiguo mar de Tetis, que en el Mioceno quedó reducido a lo que hoy es la cuenca del mar Mediterráneo (Smith *et al.*, 2004). La desecación del Mediterráneo durante la crisis salina del Mesiniense, a finales del Mioceno, dio lugar al contacto entre el continente africano y el euroasiático hace entre 5,6 y 5,3 Ma (Krijgsman *et al.*, 1999; Duggen *et al.*, 2003; Jolivet *et al.*, 2006), lo cual permitió el contacto de sus floras (Bocquet *et al.*, 1978; Caujapé-Castells &

Jansen, 2003). En el Capítulo 3 hemos inferido un ancestro común ibérico o ibero-norteafricano para la sect. *Versicolores*. El acercamiento de las placas Africana y Euroasiática hacia finales del Mioceno y la desecación del Mediterráneo en el Mesiniense podrían haber favorecido el establecimiento de un área ibero-norteafricana ya en ese ancestro común de la sect. *Versicolores*, o bien en el ancestro común de la subsect. *Versicolores*. Con la apertura del estrecho de Gibraltar y el rellenado de la cuenca Mediterránea en el límite Mioceno-Plioceno (hace 5,3 Ma; García-Castellanos *et al.*, 2009), los dos continentes quedaron nuevamente separados, lo que habría dado lugar a fenómenos de vicarianza en algunos grupos de plantas (Caujapé-Castells & Jansen, 2003; Cano-Maqueda *et al.*, 2008). En el caso de nuestro grupo de estudio, la primera divergencia dentro de la subsect. *Versicolores* podría haber estado relacionada con ese rellenado de la cuenca. Este evento creó la barrera biogeográfica responsable del aislamiento de dos clados, uno ibérico y otro ancestralmente norteafricano, que desde el evento de cladogénesis se diversificaron paralelamente (Capítulo 3, Capítulo 4). Desde la formación de esta barrera marina, la dispersión a larga distancia ha sido necesaria para la colonización entre Eurasia y África (Guzmán & Vargas, 2009; Fernández-Mazuecos & Vargas, 2010). Así, mientras que el clado ibérico de la subsect. *Versicolores* habría permanecido exclusivamente distribuido en la península Ibérica desde su origen hasta la actualidad (salvo por la colonización del sur de Francia por parte de *L. spartea*), varias especies del clado norteafricano fueron capaces, durante el Cuaternario, de colonizar distintos puntos del sur de Europa desde el norte de África (Capítulo 3). Poblaciones de *L. pedunculata* y *L. gharbensis* se han establecido en la península Ibérica, dando lugar a patrones de distribución ibero-norteafricanos, mientras que poblaciones de *L. multicaulis* se han establecido en Sicilia y el sur de la península Itálica, y *L. hellenica* se ha originado en el extremo sur de Grecia a partir de ancestros norteafricanos. Las semillas de las distintas especies de la sect. *Versicolores* parecen muy similares en cuanto a su capacidad dispersiva (Sutton, 1988). Una vez más parece que los mecanismos de dispersión han sido menos determinantes que la idoneidad de los hábitats a colonizar (Rodríguez-Sánchez *et al.*, 2008). Los repetidos eventos exitosos de dispersión a larga distancia a través de la barrera del mar Mediterráneo se relacionan, probablemente, con la expansión de los ambientes mediterráneos, a los que las linarias bífidas están bien adaptados, al igual que otros linajes de angiospermas en los que se han encontrado patrones semejantes de colonización posterior a la crisis del Mesiniense (Guzmán & Vargas, 2009; Fernández-Mazuecos & Vargas, 2010).

2. *El establecimiento del clima mediterráneo*

A lo largo de la era Terciaria o Cenozoica, las condiciones climáticas de la región que nos ocupa se aridificaron progresivamente, desde el clima tropical o subtropical del Paleoceno-Eoceno hasta las condiciones más áridas de finales del Mioceno (Thompson, 2005). Ya en el Plioceno, hace unos 2,8 Ma, se estableció la estacionalidad típica del clima mediterráneo, con sequía estival (Suc, 1984). Desde entonces proliferó la vegetación esclerófila típicamente mediterránea, y muchos linajes de plantas, antes adaptados a climas subtropicales, se adaptaron a las nuevas condiciones xéricas (Suc, 1984; Thompson, 2005). La región Mediterránea constituye en la actualidad uno de los puntos calientes (o *hotspots*) de biodiversidad del planeta (Myers *et al.*, 2000). Se está documentando la radiación de un creciente número de linajes de plantas tras el establecimiento del clima mediterráneo, por ejemplo en los géneros *Cistus* (Guzmán *et al.*, 2009), *Dianthus* (Valente *et al.*, 2010) y *Erodium* (Fiz-Palacios *et al.*, 2010). Es el caso también de *Linaria* sect. *Versicolores* (Capítulo 3, Capítulo 4) y de, al menos, otro de los clados principales del género, *Linaria* sect. *Supinae* (Blanco-Pastor *et al.*, 2012). Los dos clados de *Linaria* estudiados hasta el momento se diversificaron principalmente en el Cuaternario, tras el establecimiento del clima mediterráneo, lo cual es congruente con las condiciones mediterráneas a las que típicamente están adaptadas sus especies.

3. *La sucesión de periodos glaciares e interglaciares en el Cuaternario*

En el inicio del Pleistoceno, hace 2,6 Ma (Gibbard *et al.*, 2010), se estableció la actual sucesión de periodos fríos (glaciaciones) y cálidos (periodos interglaciares) (Paillard, 1998; Lisiecki & Raymo, 2007). Esto ha dado lugar a cambios cíclicos en la distribución de las especies a lo largo del Cuaternario, los cuales generan patrones genéticos reconocibles estudiados en el marco teórico de la filogeografía (Bennett & Provan, 2008; Avise, 2009; Stewart *et al.*, 2010). En particular, las penínsulas del sur de Europa (entre ellas la península Ibérica) sirvieron de refugio para muchos táxones europeos durante las glaciaciones gracias a su clima relativamente benigno (Comes & Kadereit, 1998; Taberlet *et al.*, 1998; Hewitt, 2000). Desde ellas se inició, en muchos casos, la recolonización del continente europeo durante los periodos interglaciares. De la misma forma, para táxones mediterráneos, determinadas áreas favorables de esas penínsulas también habrían servido de refugio, lo que habría dado lugar a patrones filogeográficos complejos (Gómez & Lunt, 2006; Nieto-Feliner, 2011). En esta memoria, la influencia de los

ciclos de periodos glaciares e interglaciares del Cuaternario se ha evaluado para una especie concreta, *L. elegans* (Capítulo 5). Para esta especie, los estudios anteriores (Capítulo 3, Capítulo 4) habían inferido una divergencia relativamente antigua (> 1 Ma) respecto a su especie hermana (*L. nigricans*), por lo que constituye un caso adecuado para el estudio de los efectos de los últimos ciclos climáticos. Se han puesto a prueba tres hipótesis para explicar la historia de la distribución de *L. elegans* en un anillo de montañas en la mitad norte de la península Ibérica. Los análisis filogeográficos y de modelización de la distribución efectuados indican la presencia de dos refugios glaciares principales, uno situado en el noroeste de la Península y otro en el oeste del Sistema Central, que divergieron durante las glaciaciones de Riss o Mindel, hace más de 100.000 años. Las poblaciones actualmente presentes en el Sistema Ibérico parecen proceder de una colonización muy reciente desde el Sistema Central, posiblemente posterior a la última glaciación (Würm). La actual distribución en anillo de *L. elegans* se explica, por tanto, por una supervivencia recurrente en refugios glaciares situados en zonas occidentales de influencia oceánica (y no a recurrentes eventos de migración altitudinal), seguida de recolonización del anillo de montañas durante los periodos interglaciares como el actual (Capítulo 5). Este resultado apoya la idea de que el gradiente climático oceánico-continental tuvo relevancia en la determinación de la localización de los refugios glaciares de ciertas especies (Stewart *et al.*, 2010). Acusadas fluctuaciones de distribución podrían también haber ocurrido en otras especies de la sect. *Versicolores*, pero al tratarse de especies de origen muy reciente en la mayoría de los casos, se requerirían marcadores moleculares más variables para desentrañar su historia en el Cuaternario (véase Apéndice 4).

4. *El papel determinante de los polinizadores en la diversidad floral*

En lo que se refiere a los factores bióticos, en el caso de las plantas con flores se ha argumentado desde antiguo el hipotético papel que habrían tenido los agentes polinizadores (especialmente los insectos) en la diversificación y en la evolución de la forma floral (Darwin, 1877; Stebbins, 1970; Kay & Sargent, 2009). La peculiar forma y los atractivos colores de la flor personada de *Linaria* y otras antirrhineas parecen claras adaptaciones a la polinización entomófila (Hill, 1909; Endress, 1994; Kampny, 1995). Por tanto, los insectos polinizadores pueden haber ejercido presiones selectivas que hayan influido en el cambio morfológico de los caracteres florales de *Linaria*. De acuerdo con nuestros resultados (Capítulo 4), el ancestro común de

la sect. *Versicolores* habría tenido una corola de tubo estrecho, espolón largo, y paladar poco desarrollado (corola escasamente ocluida), adecuada para polinizadores de probóscide larga (lepidópteros, abejas de lengua larga, dípteros). Este conjunto de caracteres es raro en *Linaria*, pero aparece en varios linajes basales del género (sect. *Macrocentrum*, sect. *Lectoplectron*) (Sutton, 1980; 1988; Capítulo 2; Apéndice 2). Tras la divergencia basal de los dos linajes principales (subsecciones) de la sect. *Versicolores*, uno de ellos (subsect. *Elegantes*) habría mantenido la forma floral ancestral en la península Ibérica, mientras que en el ancestro del otro (subsect. *Versicolores*), de distribución ibero-norteafricana (Capítulo 3), se habría producido un cambio morfológico hacia la forma típicamente melitófila (con el tubo ancho y el paladar desarrollado) de la mayoría de las especies de *Linaria* (Capítulo 4). Este cambio habría desencadenado un aumento en la tasa de diversificación en comparación con el clado de tipo morfológico ancestral. Así, al tiempo que la subsect. *Versicolores* se diversificaba profusamente, un solo evento de especiación (sin tener en cuenta posibles extinciones) habría dado lugar a las dos únicas especies de la subsect. *Elegantes*, del tipo morfológico ancestral (Capítulo 4). La divergencia antigua (> 1 Ma) entre las dos especies de la subsect. *Elegantes* (*L. elegans* y *L. nigricans*), así como la alta diversidad genética encontrada en *L. elegans* (Capítulo 5), en contraste con su uniformidad morfológica (como muestra el hecho de que no se reconocen táxones infraespecíficos; Sáez & Bernal, 2009), se pueden relacionar con la baja tasa de diversificación de este tipo morfológico. De hecho, la similitud morfológica entre *L. elegans* y *L. nigricans* es muy notable (al contrario que sus requerimientos ecológicos, totalmente contrapuestos), lo que indica una estasis morfológica (Wake *et al.*, 1983) al menos desde el ancestro de la subsect. *Elegantes*. Esta estasis es particularmente notable si la comparamos con la gran diversidad morfológica del clado hermano, la subsect. *Versicolores* (Viano, 1978b, c; Sutton, 1988). La explicación más aceptada para casos de estasis evolutiva implica la acción de la selección estabilizadora de un fenotipo óptimo constante (Charlesworth *et al.*, 1982; Estes & Arnold, 2007). Se requeriría más investigación para confirmar que ésta explicación es válida para el caso que nos ocupa.

Los ancestros de los dos grandes clados de la subsect. *Versicolores*, el ibérico y el norteafricano, habrían tenido flores de tubo ancho y paladar desarrollado. Estos caracteres se habrían mantenido en la mayor parte de las especies que evolucionaron en ambas regiones en sucesivos

eventos de especiación. Por el contrario, en unas pocas especies, tanto de la península Ibérica como del norte de África, habrían evolucionado flores de tubo estrecho, similares a las del ancestro común de la sección y a las del linaje más basal (la subsect. *Elegantes*). Sería éste, por tanto, un caso de reversión evolutiva (Porter & Crandall, 2003) que ha ocurrido en varios linajes de forma convergente (Scotland, 2011). Los dos tipos principales de flor en la sect. *Versicolores* supondrían óptimos evolutivos (Butler & King, 2004) que difieren en el modo de carga de polen por parte de los polinizadores: en la probóscide de insectos de probóscide larga (lepidópteros, abejas o dípteros) para las flores de tubo estrecho; y en la parte dorsal del tórax (*scutum*) de distintos tipos de abejas para las flores de tubo ancho. Aunque la estrategia de tubo estrecho ha aparecido repetidamente, tanto en la península Ibérica como en el norte de África, su éxito evolutivo en términos de especiación es reducido en comparación con la estrategia de tubo ancho en la subsect. *Versicolores* (Capítulo 4). Esto es congruente con la escasa diversificación de la subsect. *Elegantes*, que también presenta flores con tubo estrecho. Nuestros análisis sugieren la acción de mecanismos de "selección de especies" (Rabosky & McCune, 2010) dependiente de caracteres que restringen el acceso de los polinizadores a tres niveles: la longitud del espolón (alejamiento del néctar), la anchura del tubo floral (penetración de la cabeza y el tórax) y el desarrollo del paladar (oclusión desde el exterior). Así, las asimetrías que se encuentran en la distribución de estos caracteres en las especies actuales (p.ej. siete táxones de tubo estrecho y paladar poco desarrollado, frente a 22 de tubo ancho y paladar desarrollado) no se explican por diferencias en las tasas de cambio entre estados de carácter, sino por diferencias en las tasas de diversificación asociadas a tales estados (Maddison *et al.*, 2007; FitzJohn *et al.*, 2009; FitzJohn, 2010). Los mecanismos causantes de estas diferencias y de la consiguiente selección de especies son actualmente objeto de investigación y debate (FitzJohn, 2010; Rabosky & McCune, 2010). Para el caso de los caracteres florales, parece razonable la implicación de los polinizadores (Smith, 2010). Las diversas y cambiantes oportunidades para la diversificación proporcionadas por la fauna de polinizadores tienen posiblemente un papel importante en la generación de los patrones encontrados.

Perspectivas futuras

La investigación presentada en esta memoria sugiere una serie de posibles líneas de investigación que en el futuro permitirían profundizar en los patrones y procesos evolutivos del género *Linaria*, tal y como se describe a continuación.

Sistemática y filogenia

Para la obtención de una mayor resolución de las relaciones filogenéticas, se necesitará la secuenciación de un cierto número de genes nucleares de bajo número de copias (Li *et al.*, 2008; Zimmer & Wen, 2012). Análisis basados en simulaciones de coalescencia (Maureira-Butler *et al.*, 2008; Blanco-Pastor *et al.*, 2012) permitirán determinar las causas de las posibles incongruencias entre marcadores, y en particular el papel que pueda haber jugado la hibridación en el origen de determinados táxones. Asimismo, un mayor muestreo de especies de la sect. *Linaria* permitirá determinar el estatus evolutivo de este grupo y de la estrechamente emparentada sect. *Speciosae*. Para casos en los que la delimitación de especies es incierta, se han desarrollado recientemente métodos para el contraste de hipótesis basados en datos multilocus y en la teoría de la coalescencia (Yang & Rannala, 2010). La aplicación de estos métodos permitiría aclarar el estatus de especies politípicas de la sect. *Versicolores*, como *L. multicaulis* o *L. viscosa*.

Biogeografía

Los patrones biogeográficos y de colonización a gran escala del género *Linaria* no se han abordado en esta memoria doctoral. Análisis biogeográficos aplicados a la filogenia completa del género permitirán inferir el tiempo y dirección de los eventos de dispersión entre la región Mediterránea y otras regiones (América, resto de Eurasia) y, dentro de la región Mediterránea, entre el sur de Europa y el norte de África (de forma similar a lo efectuado para la sect. *Versicolores*). También sería interesante abordar, mediante análisis comparativos, el papel de los distintos tipos de semilla (alada y áptera) como adaptaciones para la dispersión y la colonización de nuevas áreas.

Evo-devo

En los últimos años se ha empezado a estudiar la base genética y ontogenética de algunos de los caracteres de la flor de *Linaria* (Cubas *et al.*, 1999; Galego & Almeida, 2007; Box *et al.*, 2011). Junto con la gran cantidad de información disponible para *Antirrhinum*, que es uno de los principales organismos modelo para la biología del desarrollo dentro de las angiospermas (Schwarz-Sommer *et al.*, 2003), esto permitirá llevar a cabo estudios en los que se evalúen en detalle los cambios genéticos y ontogenéticos asociados a la variabilidad morfológica del género *Linaria*. Así, por ejemplo, los diferentes patrones de coloración de la sect. *Versicolores* parecen derivados de cambios genéticos diferentes a los que actúan en *Antirrhinum* (Fernández-Mazuecos *et al.*, datos sin publicar; Whibley *et al.*, 2006). Otros caracteres interesantes a estudiar son la longitud del espolón y la anchura del tubo floral (Capítulo 4). La aplicación de las técnicas de la biología evolutiva del desarrollo o *evo-devo* permitiría determinar, por ejemplo, si la aparición repetida de flores de tubo estrecho en la sect. *Versicolores* es un caso de paralelismo (con el reclutamiento de los mismos mecanismos genéticos y del desarrollo) o sólo de convergencia (Scotland, 2011).

Macroevolución

En el Capítulo 4 se han analizado los patrones macroevolutivos de algunos caracteres florales en la sect. *Versicolores*. La disponibilidad de una filogenia de todo el género suficientemente completa y bien apoyada permitirá el análisis macroevolutivo de estos y otros caracteres en un marco filogenético más amplio. De este modo se podrá confirmar si los patrones encontrados para *Versicolores*, como la baja tasa de diversificación asociada a la flor de tubo estrecho, se mantienen para el resto del género.

Microevolución

Para la comprensión de los procesos evolutivos en los estadios incipientes, también es fundamental el estudio de las presiones selectivas que favorecen la fijación de determinados alelos y rasgos en las poblaciones. En *Linaria* son especialmente interesantes las presiones selectivas ejercidas por los polinizadores sobre caracteres que restringen el acceso de éstos al néctar, como la longitud del espolón, la anchura del tubo o el desarrollo del paladar. El estudio microevolutivo de poblaciones en las que estos caracteres sean variables permitirá una mejor

comprensión de su papel como posibles adaptaciones relacionadas con los polinizadores (Gómez *et al.*, 2006). Asimismo, pese a que no parece haber una alta especificidad en las relaciones planta-polinizador a nivel de especie, sería interesante explorar la existencia de procesos de coevolución a nivel de poblaciones (Pauw *et al.*, 2009).

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Conclusiones

Conclusions

1. According to our phylogenetic analyses, American toadflax species formerly included in the genus *Nuttallanthus* should be included in *Linaria*. Given their placement within the *Linaria* clade, this species assemblage should be treated as *Linaria* sect. *Lectoplectron*.

2. The genus *Linaria* (c. 150 species), including sect. *Lectoplectron*, is a monophyletic group within the tribe Antirrhineae.

3. The evolutionary hypothesis of a basal divergence between two major *Linaria* lineages, one of them with winged seeds, and the other with wingless seeds –as proposed by Viano– is not supported by our phylogenetic results. On the contrary, the seed wing appears to have recurrently evolved from wingless ancestors.

4. Phylogenetic analyses support the naturalness of the following sections of *Linaria*: *Versicolores*, *Macrocentrum*, *Pelisserianae* and *Lectoplectron*.

5. The sect. *Diffusae* is probably polyphyletic. It would be appropriate to disintegrate it in at least two different taxonomic entities: sect. *Diffusae s.s.* and sect. *Minutiflorae*.

6. Within the sect. *Versicolores*, the subsections *Versicolores* and *Elegantes* are monophyletic and sister to each other.

7. The most recent common ancestor of *Linaria* sect. *Versicolores* has been dated back to the Upper Miocene or Pliocene.

8. The biogeographic history of *Linaria* sect. *Versicolores* includes an old isolation between one northern African and two Iberian lineages, which independently diversified after the opening of the Strait of Gibraltar and the refilling of the Mediterranean Basin in the Early Pliocene (5.3 Ma BP).

9. Most diversification of *Linaria* sect. *Versicolores* occurred during the Quaternary (< 2.6 Ma BP), after the onset of the Mediterranean climate (2.8 Ma BP).

10. Within the subsect. *Versicolores*, at least four events of colonization after long-distance dispersal over the Mediterranean Sea gave rise to four intercontinental (European and African) lineages during the Quaternary (< 2.6 Ma BP). These colonization events occurred from northern Africa to southern Europe (two events to the Iberian Peninsula, one to Sicily/southern Italy and one to Greece).

11. The possible polyphyly of widely distributed complexes of cryptic species, as shown for *Linaria incarnata*, must be accounted for when designing the sampling strategy for biogeographic analyses. Otherwise, biased dispersal events may be inferred.

12. Three major types of flower morphology can be found in *Linaria* sect. *Versicolores*. These types are mainly differentiated by tube width, spur length and palate development.

13. At least two quantitative traits (tube width and spur length) have significantly affected the diversification rates of *Linaria* sect. *Versicolores*.

14. The morphological type with a wide tube and a long spur is found in a higher number of species than the one with a narrow tube and a long spur due to the significantly higher diversification rate of the former.

15. The narrow-tubed type has convergently evolved four to six times from broad-tubed ancestors in the subsect. *Versicolores*.

16. The morphological type with a wide tube and a short spur is found in a single, narrowly distributed species of *Linaria* sect. *Versicolores* (*L. clementei*). This species evolved from a long-spurred ancestor.

17. The broad-tubed and narrow-tubed morphological types seem to correspond to two divergent adaptive strategies of pollen charge by nectar-feeding insects: in the scutum for broad-tubed flowers; and in the proboscis for narrow-tubed flowers.

18. Even though bees are the main flower visitors (and putative pollinators) of many species of *Linaria* sect. *Versicolores*, some species are frequently visited by lepidopterans and dipterans. In particular, these may be important pollinators for some species with a narrow tube and a poorly-developed palate (non-occluded corolla).

19. The Iberian species *Linaria elegans* diverged from its sister species *L. nigricans* > 1 Ma BP. Since then, these two species with narrow tubes, long spurs and poorly-developed palates have survived, without relevant floral morphological differentiation, the Quaternary climatic cycles in the Iberian Peninsula. They have, however, diverged in their ecology and distribution: *L. elegans* in the northern half of the Iberian Peninsula and *L. nigricans* in a few localities of the arid south-east.

20. The current distribution of *L. elegans*, mainly in a mountain ring in the northern half of the Iberian Peninsula, is the result of the recurrent survival of this species in a minimum of two glacial refugia located in western areas with an oceanic climate (one north and one south of the Duero basin), followed by recolonization of the mountain ring during inter-glacial periods.

21. The divergence between the two main glacial refugia of *L. elegans* dates back to the Riss or Mindel glaciations (> 100,000 years BP). No evidence of significant recent gene flow between them has been found.

22. Current populations of *L. elegans* in the Iberian System are the result of a recent (probably Holocene) colonization from populations of the eastern Central System.

23. Phylogeographic reconstructions provide independent data that allow testing the reliability of different simulations of past climates (such as those of the Last Glacial Maximum) used in species distribution modelling.

24. The evolution of *Linaria* sect. *Versicolores* has been influenced by both historical abiotic factors (the history of contacts and isolation between the Eurasian and African plates, the onset of the Mediterranean climate, the Quaternary climatic cycles) and biotic factors (insect pollinators). The contribution and prevalence of each factor depends on the studied lineage.

Apéndice 1

Filogenia datada de la tribu Antirrhineae: comprobando la hipótesis de congruencia biogeográfica

A time-calibrated phylogeny of the tribe Antirrhineae: testing the biogeographic congruence hypothesis

Este tabajo se ha desarrollado en colaboración con José Luis Blanco Pastor, Isabel Liberal, Beatriz Guzmán, Emilio Cano (Real Jardín Botánico, CSIC), Luis Valente (Imperial College, London) y Alan Forrest (Royal Botanic Garden, Edinburgh)

Los datos presentados en este apéndice forman parte de una publicación actualmente en proceso de revisión en *Systematic Biology*:

Vargas P, Valente LM, Blanco-Pastor JL, Liberal I, Guzmán B, Cano E, Forrest A, Fernández-Mazuecos M. Testing biogeographic congruence of palaeofloras using Bayesian molecular phylogenetics. Enviado.

Abstract

The tribe Antirrhineae is one of the plant groups that better illustrates a New World (14 genera) and Old World (15 genera) disjunction. This group has been hypothesized to be part of the Tertiary Madrean-Tethyan palaeoflora. Here we present the first dated phylogenetic analysis of the tribe Antirrhineae, based on plastid DNA sequences and calibrated using five fossils of order Lamiales and a secondary basal calibration. The biogeographic congruence hypothesis —that is similar spatio-temporal pattern of geographical distribution across lineages— is tested by means of Bayesian inference in concert with parsimony and maximum likelihood methods. Our analyses revealed a synchronous (Miocene) colonization of America by four independent lineages of Antirrhineae. This result implies the first example of high biogeographic congruence within the same plant group. We argue that explicit testing of the biogeographic congruence hypothesis, using additional angiosperm disjunctions, will provide solid evidence for the origin and evolution of the Madrean-Tethyan palaeoflora and other ancient floras.

Introduction

Species distributions are the result of the success of colonization processes. Biogeographers conceive a continuous process of dispersal, establishment and extinction to account for the present-day ranges of about 250,000 species of angiosperms (Cox, 2001). The likelihood of observing similar species distributions is low given the high number of variables involved, for example dispersal mechanisms, habitat availability, mutualistic interactions and competition. Nevertheless, some species share similar distributions inasmuch as they form community assemblages as a result of sharing similar biotic and abiotic preferences (Phillips *et al.*, 2006) and biogeographic histories (Ronquist & Sanmartín, 2011). The Earth harbours plant communities distributed in six floristic kingdoms that reflect colonization, differentiation and extinction processes over time (Takhtajan, 1986; but see Cox, 2001 for discussion). Scholars have studied the origin of community assemblages to explore whether they are primarily the result of ancient sharing of species distributions or recent species coexistence. Accordingly, examination of the occurrence of taxa in two or more territories needs to consider not only spatial variables (geography), but also temporal components in order to interpret congruence of historical processes (Cunningham & Collins, 1994; see Cox, 2001). The pattern of geographical distribution shared by two or more lineages and originated during the same period is known as biogeographic congruence, whereas biogeographic pseudocongruence indicates that the common pattern originated at different times (Page, 1990; Cunningham & Collins, 1994; Wen, 1999; Donoghue & Moore, 2003). The lure of understanding fragmented distributions has historically led biogeographers to recognize different categories of intercontinental disjunctions (Wen & Ickert-Bond, 2009): seven main categories were proposed by Raven (1972) and 16 by Thorne (1972) to account for biogeographically discontinuous distributions. Once plant groups sharing discontinuous distributions are identified, a temporal framework is required to unveil whether disjunctions occurred synchronously (Donoghue & Moore, 2003). Fossil records help reconstruct distribution patterns in a temporal scale allowing researchers to trace the history of angiosperms. However, their fragmented deposition results in temporal discontinuities. The advent of molecular phylogenetics can give a reliable reconstruction of the tempo of colonization and evolution, especially when complemented by chorology, paleontology and paleoecology. Phylogenetic reconstructions not only help elucidate common ancestry, but also investigate historical biogeography by means of parsimony, maximum-likelihood and Bayesian

methods (Ree & Smith, 2008; Yu *et al.*, 2010; Ronquist & Sanmartín, 2011). New tools for reliably estimating divergence times using the fossil record and DNA sequences (Drummond & Rambaut, 2007) additionally provide a temporal framework to biogeographic disjunctions. As a result, an increasing number of coincidental patterns of historical biogeography of animals and plants have been detected (Donoghue & Smith, 2004). These new approaches offer the opportunity to test whether similar biogeographic patterns are the result of asynchronous events of dispersal or vicariance (pseudocongruence) or reflect historical sharing of such processes (congruence) (Donoghue & Moore, 2003).

Some of the most intriguing disjunctions are the north temperate discontinuous distributions, which include lineages distributed in Asia, Europe and North America (Raven, 1972). It has been suggested that the fragmentation of the two floristic regions (Mediterranean and Californian) that share a summer-drought climate in the same period of the year (May-September) may be the result of a vicariance process and ecological maintenance of the Madrean-Tethyan palaeoflora since the early Tertiary (Axelrod, 1975; Raven & Axelrod, 1978). Both the barrier of the Atlantic Ocean for land plants and the summer-humid climate of south-eastern North America make any current floristic connection unlikely. This temporal hypothesis has been challenged by molecular phylogenetics primarily in the last decades (see Manos & Donoghue, 2001). Wen & Ickert-Bond (2009) comprehensively reviewed molecular phylogenies addressing the current disjunction of Madrean-Tethyan plant groups. They reviewed floristic relationships and divergence times between lineages of the two regions and concluded that (i) direct vicariance appears to be unlikely given the upper boundary estimate for this hypothesis (25 Ma); (ii) convergent evolution may have reinforced the floristic similarity; and (iii) a directionality of the migration/dispersal from the Old to the New World is predominant. However, they recognized that most of the 14 study cases of angiosperms used for the analysis lacked fossil records and explicit methodology to reconstruct divergence times in a phylogenetic framework. The question therefore remains as to whether a high degree of biogeographic congruence occurs in cases of Old and New World disjunctions, and to what extent coincident timing of colonization reveals crucial paleogeographic and paleoclimatic events.

The objective of this study is to date lineage divergence to account for the origin of the Madrean-Tethyan disjunction of a natural group of angiosperms (tribe Antirrhineae). It has

been suggested that the Antirrhineae (snapdragons) is one of the best candidates to test the biogeographic congruence hypothesis (hereafter BCH) because at least three lineages displayed a similar Madrean-Tethyan pattern (Raven & Axelrod, 1978; Vargas *et al.*, 2004; Wen & Ickert-Bond, 2009). To explore biogeographic patterns, we used an approach to a parsimony-based method of ancestral range reconstruction that accounts for phylogenetic uncertainty (Yu *et al.*, 2010) and a maximum-likelihood modelling of range inheritance scenarios at cladogenesis events (Ree & Smith, 2008). Finally, the concept of biogeographic congruence is revisited to define particular predictions associated with geological periods. Our ultimate goal is to infer whether the intercontinental disjunctions of Antirrhineae reflect historical processes that may have been facilitated by temporal windows of opportunity for colonization.

Materials and methods

Testing the BCH

The geographical and evolutionary pattern shared by two or more lineages that occur in the same territory at a similar period of time is known as biogeographic congruence (Cunningham & Collins, 1994; Wen, 1999; Donoghue & Moore, 2003). Phylogenetic inference helps provide the evidence required to support the BCH for the Madrean-Tethyan flora in general, and for the Antirrhineae in particular: (i) common ancestry of plants involved in the Old-New World disjunction as hypothesized based on taxonomy; (ii) an unequivocal sister relationship between Mediterranean and Californian clades; (iii) availability of appropriate fossil-based calibration points for phylogenetic dating; (iv) identification of two or more lineages displaying the same biogeographic pattern; and (v) sharing of the same window of colonization in a temporal scale. Common ancestry and sister-group relationships are inferred based on monophyly and biphyly of DNA sequences, respectively. Molecular clock techniques, coupled with the fossil record, furnish detailed information of absolute timing of lineage splits. Finally, new biogeographic approaches estimate the directionality of lineage connections. Here we use this approach to test the BCH in the Antirrhineae.

Sampling strategy and DNA sequencing

In the Antirrhineae and most angiosperms, plastid DNA is usually inherited by ovules (Corriveau & Coleman, 1988), and therefore preferred to reconstruct seed colonization (see Fernández-Mazuecos & Vargas, 2011; Chapter 3). A total of 60 accessions of plastid (*ndh*F) sequences were herein included (Table 1). These represent most genera (27) within the Antirrhineae (Sutton, 1988). In addition to Sutton's (1988) taxonomic treatment, we included two more genera (*Pseudomisopates*, *Nanorrhinum*) described thereafter. We failed to find and include material of two New World genera (*Epixiphium*, *Holmgrenanthe*). Naturalness of the Antirrhineae genera has been partially inferred (Vargas *et al.*, 2004). In order to analyze one accession per genus, 19 sequences of the Antirrhineae were newly generated for this study and 8 were retrieved from the GenBank database. Outgroup samples were also obtained from the GenBank database, and were chosen to represent both closely related lineages (Albach *et al.*, 2005) and more distant families (Schäferhoff *et al.*, 2010) for which reliable fossils are available. The outgroup consisted of 33 sequences of the Lamiales, including the families Plantaginaceae, Bignoniaceae, Acanthaceae, Orobanchaceae, Lamiaceae, Verbenaceae, Pedaliaceae, Scrophulariaceae, Gesneraniaceae, Calceolariaceae, Oleaceae and Plocospermataceae.

Genomic DNA was extracted using the Plant DNeasy kit (Qiagen Inc.) according to manufacturer's recommended protocols. The plastid *ndh*F gene was amplified via Polymerase Chain Reaction (PCR) using primers #1 and #2112R of Olmstead & Reeves (1995) and sequenced with the PCR primers and internal primers #972F and #1318R of Olmstead & Sweere (1994). PCR was undertaken in a total volume of 25 µl including 1x PCR buffer, 3 mM $MgCl_2$, 0.16 mM dNTPs, 1 mg/ml bovine serum albumin, 0.5 µM of each primer, 1.5 U EcoTaq (Bioline) and 1 µl of DNA template. The thermocycling profile was: initial denaturation at 95ºC for 2 minutes, followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 52ºC for 2 minutes and extension at 72ºC for 1 minute 30 secs, followed by a final extension at 72ºC for 10 minutes and storage at 4ºC. Amplification was confirmed via agarose gel electrophoresis before sequencing with BigDye Terminator v3.1 cycle sequencing kit on a 3730 DNA analyzer (Applied Biosystems Inc.). Sequences for each accession were edited and combined into a single contig using Sequencher 3.7 (Applied Biosystems Inc.).

Phylogenetic analysis

The sequences were aligned using MAFFT v.6 (Katoh *et al.*, 2002) with minor adjustments made by visual inspection. Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) analyses were conducted. To determine the optimal model of sequence evolution that best fits the sequence data (GTR+I+G), the Akaike information criterion (AIC) was implemented using jModelTest 0.1.1 (Posada, 2008). BI was performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). We ran two sets of four Markov chains for 10 million generations, sampling every 1000 generations. Parameters of both runs converged on the same stable distribution after c. 100,000 generations, as seen in Tracer 1.4 (Rambaut & Drummond, 2007). Therefore, trees for the first 10% generations were discarded as burn-in. ML analysis was performed using PhyML 3.0 (Guindon & Gascuel, 2003) including the model parameters previously obtained with jModelTest. Node supports for the ML analysis were estimated using 500 non-parametric bootstrap replicates. Parsimony analysis was run using the package TNT 1.1 (Goloboff *et al.*, 2003). An initial heuristic search was run using the Tree Bisection-Reconnection (TBR) branch-swapping algorithm with 10,000 replicates, saving two most-parsimonious trees per replicate. Trees obtained in the first search were then used to start an additional heuristic search (Ratchet and Tree drifting algorithms) (Goloboff, 1999; Nixon, 1999). Bootstrapping values were scored after resampling the matrix with 10,000 replicates.

Bayesian dating

To estimate divergence times among Lamiales lineages, including the Antirrhineae genera, we analyzed the *ndh*F matrix through a relaxed molecular-clock approach in BEAST v.1.6.1 (Drummond & Rambaut, 2007). No reliable fossils of the Antirrhineae appropriate for molecular calibration have been discovered to date (Martínez-Millán, 2010). Therefore, we employed previous molecular estimates and five Lamiales fossils to this end (Fig. 1). The basal divergence between the Oleaceae and Antirrhineae was modelled as a normal distribution with mean = 74 Ma and standard deviation = 2.5 Ma, on the basis of an estimate obtained in the most recent relaxed-clock analysis of angiosperms (Bell *et al.*, 2010). In addition, we implemented five minimum age constraints for Lamiales families and tribes based on five fossils (Table

Table 1. Material used for *ndh*F sequencing of 27 samples (genera) of the tribe Antirrhineae (including 8 samples from the GenBank). The outgroup samples of different families were taken from the GenBank, except for *Lafuentea rotundifolia*. Taxon, locality, voucher reference, GenBank accession number and bibliographic reference are given for each accession.

Taxon	Locality	Voucher	GenBank accession no.	Reference
PLANTAGINACEAE				
Lafuentea rotundifolia Lag.	Spain, Almería, Pechina	MA 705703	JN848475	This study
Tribe Antirrhineae				
Acanthorrhinum ramosissimum (Coss. & Durieu) Rothm.	Morocco, road from Ouarzazate to Zagora	J. Güemes 3285MS (VAL 41469)	JN848476	This study
Albraunia foveopilosa Speta	Iran, Khuzistan, Baghmalek-Haftgel	TARI 38909	JN848477	This study
Anarrhinum bellidifolium Desf.	Cultivated. National Botanic Garden, Dublin	VAL 145150	JN848478	This study
Antirrhinum majus L.	Spain, Barcelona, Greixer	J. Güemes JG4150	JN848479	This study
Asarina procumbens Mill.	Cultivated. Uppsala Botanical Garden	Ghebrehiwet 461 (UPS)	AJ250380	Ghebrehiwet *et al.* (2000)
Chaenorhinum origanifolium (L.) Kostel.	Spain, Huesca, Bujaruelo	J. Güemes s.n.	JN848480	This study
Cymbalaria muralis G.Gaertn., B.Mey & Scherb.	Cultivated. Uppsala Botanical Garden	Ghebrehiwet 378 (UPS)	AJ250382	Ghebrehiwet *et al.* (2000)
Galvezia fruticosa J.F.Gmel.	Perú, La Libertad, San Pedro de Lloc	Elisens 825 (OKL)	JN848481	This study
Gambelia speciosa Nutt.	Cultivated. Botanischer Garten Berlin-Dahlem	J. Güemes. 164/98 (VAL 145156)	JN848482	This study
Holzneria spicata (Korovin) Speta	Iran, Khorasan, Tobart-e Sefid	TARI 23577	JN848483	This study
Howelliella ovata (Eastw.) Rothm.	USA, California, San Luis Obispo Co.	RW Storer 453 (UPS)	AJ250385	Ghebrehiwet *et al.* (2000)
Kickxia elatine (L.) Dumort.	Cultivated. Uppsala Botanical Garden	Ryding 1210 (UPS)	AJ245816	Ghebrehiwet *et al.* (2000)
Linaria vulgaris Mill.	France, Chamonix	B. Estébanez s.n.	JN848484	This study
Lophospermum erubescens D.Don	Cultivated. Botanischer Garten Berlin-Dahlem	VAL 145154	JN848485	This study
Mabrya acerifolia (Pennell) Elisens	USA, Arizona	Elisens s.n.	JN848486	This study
Maurandella antirrhiniflora (Kunth) Rothm.	México, Guanajuato	Billiet & Jadin 6209 (MA 588497)	JN848487	This study
Maurandya scandens (Cav.) Pers.	Cultivated. Uppsala Botanical Garden	Gebrehiwet 367 (UPS)	AJ245818	Ghebrehiwet *et al.* (2000)
Misopates orontium (L.) Raf.	Spain, Valencia, Serra	J. Güemes (VAL 145155)	JN848488	This study
Mohavea confertiflora A.Heller	USA, Arizona.	Nelson 1348 (UPS)	AJ250389	Ghebrehiwet *et al.* (2000)
Nanorrhinum elegans (G.Forst.) Ghebr.	Cabo Verde	J.J. Aldasoro A9511	JN848489	This study
Neogaerrhinum kelloggii (Greene) Thieret	USA, California, Los Angeles Co.	D.M. Thompson 306 (A 257572)	JN848490	Oyama & Baum (2004)
Nuttallanthus texanus (Scheele) D.A.Sutton	USA, California, Del Mar Mesa	Breedlove 62452 (MA 494665)	JN848491	This study
Pseudomisopates rivas-martinezii (Sánchez Mata) Güemes	Spain, Ávila, Sierra de Gredos, La Serrota	J. Güemes JG1737	JN848492	This study
Pseudorontium cyathiferum (Benth.) Rothm.	USA, California	Van Devender 92-268 (AZ)	JN848493	This study
Rhodochiton atrosanguineum (Zucc.) Rothm.	Cultivated. Uppsala Botanical Garden	Gebrehiwet 375 (UPS)	AJ250390	Ghebrehiwet *et al.* (2000)
Sairocarpus nuttallianus (A.DC.) D.A.Sutton	USA, California, Del Mar Mesa	Breedlove 62454 (MA 494669)	JN848494	This study
Schweinfurthia papilionacea Boiss.	Oman, near Muscat	E 46435	JN848495	This study
Tribe Callitricheae				
Callitriche hermaphroditica L.	USA, California, Lassen Co.	Philbrick 3022 (WCSU)	L36396	Olmstead & Reeves (1995)
Tribe Cheloneae				
Collinsia grandiflora Douglas ex. Lindl.	Cultivated. Oklahoma University	Wolfe 130	AF188182	Olmstead *et al.* (2001)
Chelone obliqua L.	Cultivated. Indiana University	C.W. Morden 853 (PSU)	AF123680	Olmstead *et al.* (2001)
Penstemon sp.	Unknown	Oxelman 2338 (WTU)	AJ619565	Oxelman *et al.* (2005)
Tribe Digitalideae				
Digitalis purpurea L.	Unknown	KJ Kim 13943 (YNUH)	AF130150	Olmstead *et al.* (2000)
Digitalis grandiflora Mill.	USA, Colorado, Boulder, University of Colorado campus	Olmstead 92-115 (WTU)	L36399	Olmstead & Reeves (1995)
Isoplexis canariensis (L.) Loudon	Spain, Tenerife, Anaga Peninsula	Thulin 9945 (UPS)	AJ617597	Oxelman *et al.* (2005)

Table 1. Continued

Tribe Globularieae				
Globularia nudicaulis L.	Cultivated. Royal Botanic Gardens, Kew	082-57-08201	AF123681	Olmstead *et al.* (2001)
Tribe Gratioleae				
Bacopa caroliniana B.L.Rob.	Unknown	C.W. dePamphilis 90.137 (PAC)	AF123677	Olmstead *et al.* (2001)
Gratiola pilosa Michx.	Unknown	C.W. dePamphilis 90.34	AF188183	Olmstead *et al.* (2001)
Stemodia suffruticosa Kunth	Ecuador, Zamora-Chinchipe, Parque Nacional Podocarpus	J.E. Madsen 85727 (MO)	EF527455	Estes & Small (2008)
Tribe Plantagineae				
Plantago lanceolata L.	Unknown	KJ Kim 13790 (YNUH)	AF130151	Olmstead *et al.* (2000)
Tribe Veroniceae				
Veronica persica Poir.	USA, Colorado, Boulder, University of Colorado campus	Olmstead 92-144 (WTU)	L36419	Olmstead & Reeves (1995)
ACANTHACEAE				
Thunbergia alata Sims	Unknown	OXF	U12667	Scotland *et al.* (1995)
BIGNONIACEAE				
Catalpa bignonioides Walt.	USA, Colorado, Boulder, University of Colorado campus	Olmstead 92-99 (WTU)	L36397	Olmstead & Reeves (1995)
Kigelia africana (Lam.) Benth.	Cultivated. Waimea Botanical Garden, Kenya	Rica s.n. 74s980	AF102632	Spangler & Olmstead (1999)
CALCEOLARIACEAE				
Calceolaria sp.	Unknown	C.W. dePamphilis 90.68 (PAC)	AF123679	Olmstead *et al.* (2001)
GESNERIACEAE				
Nematanthus hirsutus (Mart.) Wiehler	Cultivated. Marie Selby Botanical Garden	na	L36404	Olmstead & Reeves (1995)
Streptocarpus holstii Engl.	Cultivated. University of Michigan Matthaei Botanical Garden	na	L36415	Olmstead & Reeves (1995)
LAMIACEAE				
Lamium purpureum L.	Unknown	na	U78694	Wagstaff *et al.* (1998)
Teucrium fruticans L.	Cultivated. Ohio University	P Cantino 1287 (BHO)	U78686	Wagstaff *et al.* (1998)
OLEACEAE				
Fraxinus chinensis Roxb.	Cultivated, Arnold Arboretum	8161-A	DQ673275	Lee *et al.* (2007)
Jasminum mesnyi Hance	Cultivated. Royal Botanic Garden Edinburgh	19697040	DQ673267	Lee *et al.* (2007)
Ligustrum vulgare L.	Cultivated. Arnold Arboretum	na	AF130164	Olmstead *et al.* (2000)
Olea europaea L.	Cultivated. University of Conneticut	19850974	DQ673278	Lee *et al.* (2007)
OROBANCHACEAE				
Bartsia alpina L.	Austria, Styria	C.W. dePamphilis 93.37 (PSU)	AF123678	Olmstead *et al.* (2001)
Pedicularis foliosa L.	Austria, Styria	Wetschnig (PSU)	AF123689	Olmstead *et al.* (2001)
PEDALIACEAE				
Sesamum indicum L.	Cultivated	na	L36413	Olmstead & Reeves (1995)
PLOCOSPERMATACEAE				
Plocosperma buxifolium Benth.	Unknown	Salinas 8050 (MEXU)	AJ011985	Oxelman *et al.* (1999)
SCROPHULARIACEAE				
Buddleja davidii Franch.	Cultivated. New York Botanical Garden	na	AF130143	Olmstead *et al.* (2000)
Scophularia californica Cham. & Schltdl.	Unknown	C.W. DePamphilis SS20 (PAC)	L36411	Olmstead & Reeves (1995)
VERBENACEAE				
Verbena bracteata Cav. ex. Lag. & Rodr.	USA, Colorado, Boulder.	Olmstead 92-131 (WTU)	L36418	Olmstead & Reeves (1995)

2; Fig. 1). All of them have been considered reliable and proposed as calibration points for molecular dating in previous studies (Besnard *et al.*, 2009; Martínez-Millán, 2010; Thiv *et al.*, 2010). Fossil calibrations were placed at the stem nodes of clades, or the basal nodes of taxa, to which fossils are unambiguously assigned (Fig. 1). When necessary, clades were constrained as monophyletic based on phylogenetic results. Fossil calibrations were modelled as log-normal distributions with mean = 0, standard deviation = 1 and offset values corresponding to the upper limit of the time interval to which fossils are assigned (Table 2). The substitution rate variation was modelled using an uncorrelated lognormal distribution, and a birth-death process (Gernhard, 2008) was employed as tree prior. Four MCMC analyses were run for 20 million generations each, with a sample frequency of 1000. Chain convergence was examined in Tracer 1.4 (Rambaut & Drummond, 2007) by inspection of trace plots and effective sample sizes (ESS). The four chains were combined in LogCombiner 1.4.8 after discarding the first 10% of sampled generations as burn-in. Trees were summarized in a maximum clade credibility (MCC) tree obtained in TreeAnotator 1.4.8 and visualized in FigTree 1.3.1.

Table 2. Fossil calibration points and associated minimum age constraints employed in the relaxed-clock analysis of *ndh*F sequences of Lamiales (including Antirrhineae genera). Nodes are named as in Fig. 1.

Node	Fossil (family/tribe)	Time interval	Minimum age (Ma)	Reference
A	***Fraxinus wilcoxiana*** Berry (Oleaceae)	Middle Eocene	37.2	Call and Dilcher (1992)
B	***Catalpa rugosa*** Reid & Chandler (Bignoniaceae)	Early-Middle Oligocene	28.4	Reid & Chandler (1926)
C	***Ajuginucula smithii*** Reid & Chandler (Lamiaceae)	Early-Middle Oligocene	28.4	Reid & Chandler (1926)
D	***Gratiola tertiaria*** Łańcucka-Środoniowa (Plantaginaceae/ Gratioleae)	Miocene	5.3	Łańcucka-Środoniowa (1977)
E	***Plantaginacearumpollis*** Nagy (Plantaginaceae/Plantagineae)	Middle Miocene	11.6	Nagy (1963)

Ancestral area reconstructions

We performed biogeographic reconstructions by applying two different methods. Both reconstructions were conducted delimiting two areas (Old World and New World), and ancestors were allowed to be present in both. First, we analyzed the BEAST output using S-DIVA (Yu *et al.*, 2010), an approach to the parsimony-based dispersal-vicariance analysis (DIVA; Ronquist, 1997) that accounts for the uncertainty of a Bayesian phylogenetic analysis. S-DIVA analyses were conducted following the methods of Harris & Xiang (2009) implemented in the program RASP 1.1 (Yu *et al.*, 2011). All outgroup taxa, except for *Lafuentea* (which is sister to the Antirrhineae) were pruned from the trees. We used 1000 random trees sampled after the burn-in period from the BEAST run and the pruned MCC tree as final tree.

We also performed dispersal-extinction-cladogenesis (DEC) analysis (Ree *et al.*, 2005; Ree & Smith, 2008), a parametric likelihood-based approach to reconstruct ancestral distributions. DEC analysis estimates the most likely geographic distribution of the two daughter lineages following a speciation event. Thus, whereas S-DIVA estimates the actual state at the node, DEC estimates the states of the branches emanating from a given node. DEC analysis was implemented using the software package Lagrange v2.0.1 (Ree & Smith, 2008). We set symmetric dispersal between both areas, and constant dispersal rates through time, given that the sea barrier between the Old and the New World formed in the Eocene (Axelrod, 1975) remained for the entire time frame of Antirrhineae diversification indicated by the dating analysis (see below).

Temporal congruence

To statistically evaluate the temporal congruence of the multiple Old World – New World disjunctions found in the Antirrhineae, we compared divergence time estimates for the four Old-New World lineages across the combined posterior distribution of 72,000 trees from the BEAST analysis. Available standard methods for biogeographic reconstruction (such as those here employed: S-DIVA and DEC) assume a "vicariance-mediated allopatry" scenario, thus modelling dispersal as occurring along the branches of the phylogenetic tree (Buerki *et al.*, 2011). However, a model of "dispersal-mediated allopatry", in which dispersal is immediately followed by cladogenesis, is considered to be more realistic when long-distance dispersal across

ocean barriers is involved (Clark *et al.*, 2008; Buerki *et al.*, 2011), as occurs in Antirrhineae (see below). Under this scenario, divergence times between Old and New World lineages of Antirrhineae would constitute a good estimate of colonization times.

Divergence time estimates for the four Old-New World lineages were first compared by inspection of the marginal density distributions of times to the most recent common ancestor (TMRCAs) in Tracer 1.4. Then, we obtained the posterior probability (PP) of occurrence of each divergence within each geologic epoch, as defined by the International Stratigraphic Chart 2009 (available at www.stratigraphy.org). This probability was calculated as the proportion of trees from the posterior distribution where a particular Old-New World divergence fell into the bounds of a certain geologic epoch. Finally, we calculated the PP of temporal congruence of two or more colonization events as the proportion of trees in which two, three or four Old-New World divergences occurred within the bounds of the same geologic epoch. In order to provide statistical support for the BCH, PPs above 0.50 were classified into high (0.90-1.00 PP), medium (0.75-0.90 PP) and low (0.50-0.75 PP).

Results

Phylogenetic analysis

The *ndh*F matrix contained 2086 bp and had 1047 variable sites, of which 686 were parsimony-informative. The phylogenetic analyses using 60 accessions from 27 genera of the Antirrhineae and 33 outgroup sequences revealed that the Antirrhineae form a monophyletic group with *Lafuentea* as the sister taxon (Fig. 1). All three phylogenetic analyses (BI, ML and MP) recognized three well-supported clades formed by Old-New World sister lineages, which have been named as follows (see Fig. 1): *Cymbalaria* lineage (PP = 1; ML-BS = 99%; MP-BS = 97%), *Galvezia* lineage (PP = 1; ML-BS = 100%; MP-BS = 100%) and *Linaria* lineage (PP = 1; ML-BS = 100%; MP-BS = 100%). One more Old-New World clade was less supported, the *Sairocarpus* lineage (PP = 0.66; ML-BS = 63%; MP-BS = 63%), which was included, together with the Old World *Antirrhinum* in a strongly supported lineage (PP = 1; ML-BS = 100%; MP-BS = 100%).

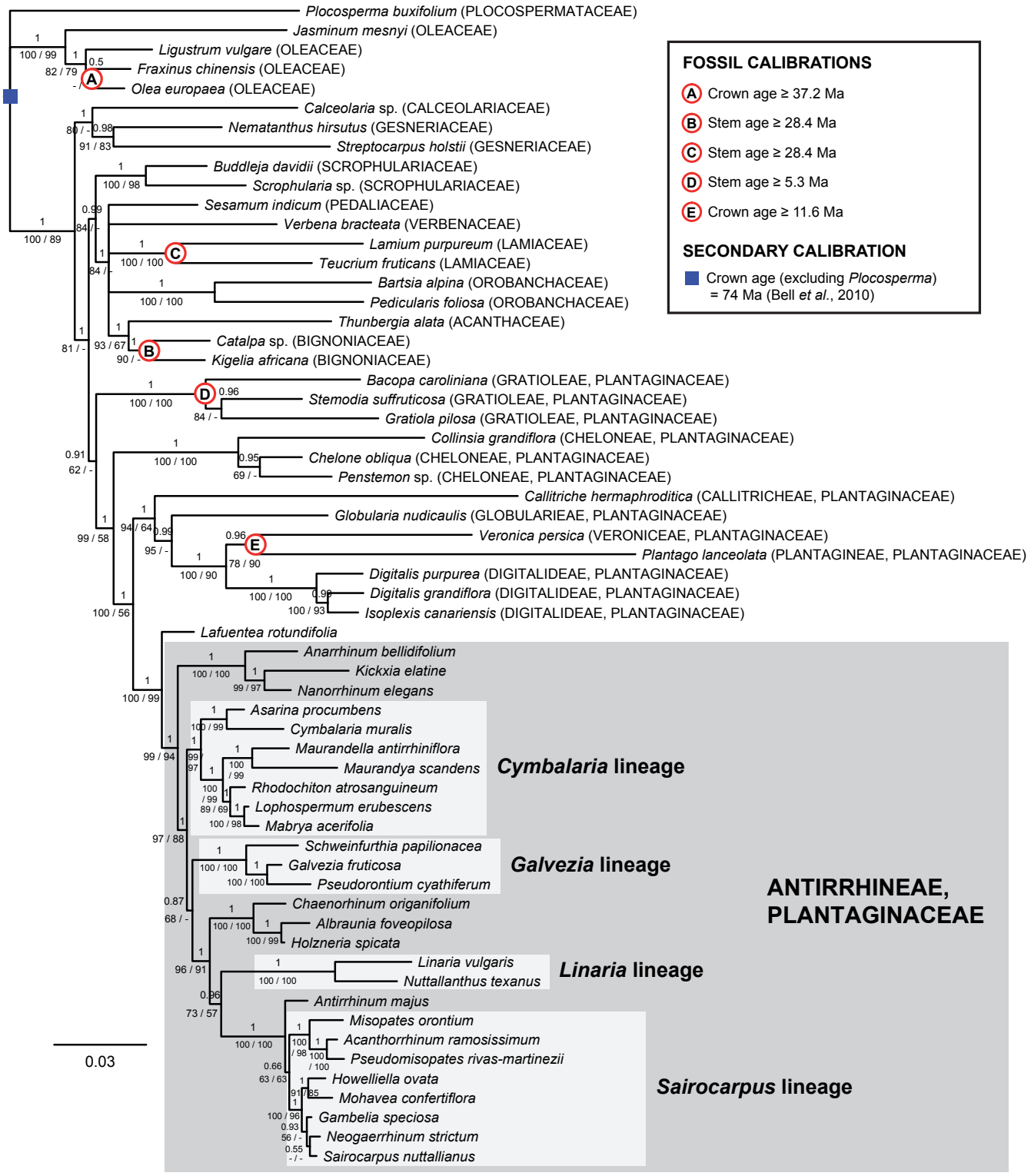


Fig. 1. Phylogenetic relationships of Lamiales (including Antirrhineae) based on *ndh*F sequences. The fifty-percent majority-rule consensus tree obtained in the MrBayes analysis is shown. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood/maximum parsimony percentage bootstrap values. Calibration points used in the dating analysis are indicated. The four Old-New World lineages discussed in the text are delimited.

Bayesian dating

The values of standard deviation of the uncorrelated lognormal relaxed clock (0.748) and coefficient of variation (0.783) for rate heterogeneity within our *ndh*F dataset indicated the presence of rate heterogeneity among lineages. Analysis with Tracer 1.4 confirmed adequate sample size, with ESS values above 300 and plots showing equilibrium after discarding the burn-in. The topology of the MCC tree (Fig. 2) was congruent with those of previous phylogenetic analyses. Diversification of major lineages of Antirrhineae (Table 3; Fig. 2) was estimated to have spanned a long period since the Oligocene. Mean ages of the four most recent common ancestors of Old-New World lineages fell within the bounds of the Miocene (Fig. 2).

Table 3. Mean posterior estimated ages and 95% highest posterior density (HPD) intervals for the nodes of the phylogeny of Antirrhineae based on relaxed molecular-clock analysis of *ndh*F sequences in BEAST (Fig. 2). Nodes are numbered as in Fig. 2. Posterior probabilities from the same analysis are also shown.

Node	Posterior probability (BEAST)	Mean age (Ma)	95% HPD interval (Ma)
1	1.00	33.9	24.8-43.1
2	1.00	30.2	22.4-39.2
3	1.00	15.8	6.8-25.0
4	1.00	10.0	3.2-17.8
5	1.00	27.7	19.9-35.8
6	1.00	20.8	12.4-29.7
7	1.00	10.1	2.6-18.4
8	1.00	13.5	6.7-20.8
9	1.00	8.3	3.2-14.1
10	1.00	8.0	2.2-14.4
11	1.00	2.6	0.4-5.7
12	0.77	26.1	18.7-34.2
13	1.00	13.2	5.6-21.7
14	1.00	6.7	1.7-13.0
15	1.00	22.9	15.8-30.3
16	1.00	10.6	4.2-18.2
17	1.00	3.3	0.7-7.0
18	0.92	20.3	13.3-27.3
19	1.00	9.8	3.9-16.5
20	1.00	11.1	6.2-16.8
21	0.54	10.1	5.3-15.3
22	1.00	6.6	2.8-11.0
23	1.00	3.0	0.8-5.9
24	1.00	5.7	2.4-9.5
25	1.00	3.9	1.3-7.2
26	1.00	2.7	0.6-5.3
27	0.46	-	-

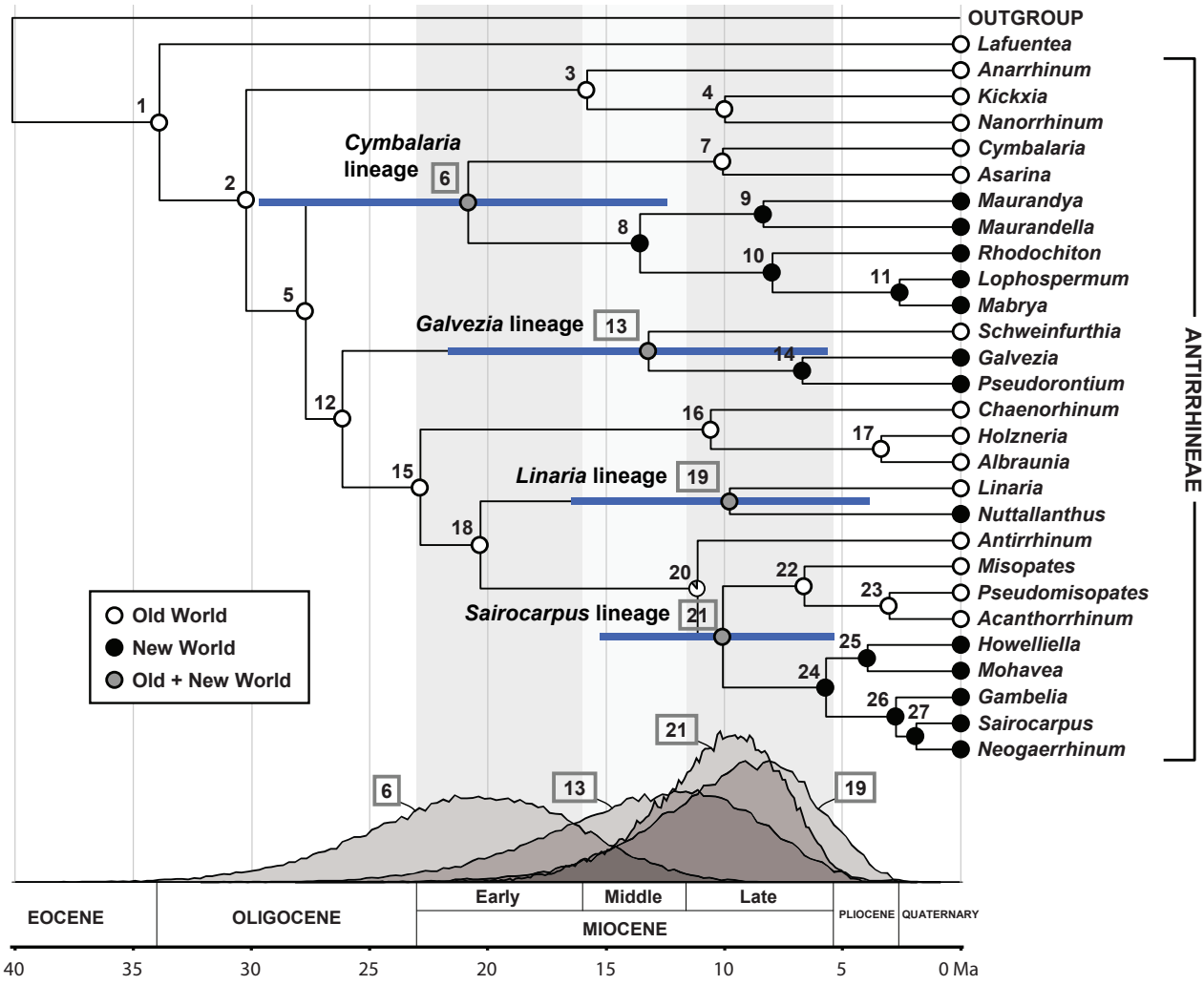
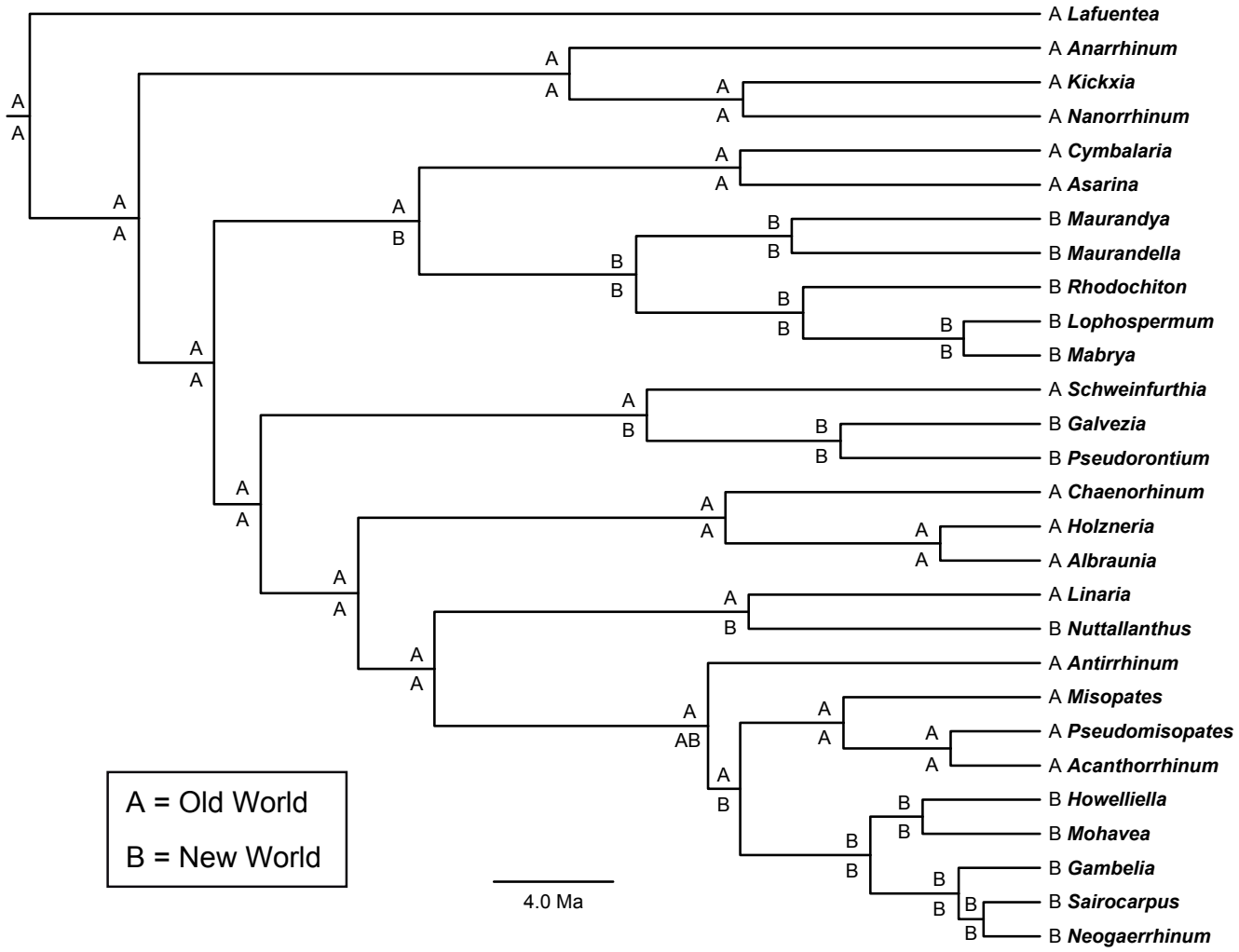


Fig. 2. Molecular dating analysis and S-DIVA biogeographic reconstruction of Antirrhineae based on *ndh*F sequences. The maximum clade credibility tree produced by relaxed molecular-clock analysis is shown. Outgroup lineages (except for *Lafuentea*) have been collapsed for clarity. Nodes are numbered as in Table 3. Pie charts at nodes represent marginal probabilities for ancestral areas as inferred by S-DIVA analysis. Node bars in blue represent the 95% highest posterior density intervals for TMRCAs of four Old-New World lineages. Marginal densities of TMRCAs of the same four lineages are represented along the time scale.

Ancestral area reconstructions

Biogeographic analyses not only supported an Old-New World common ancestry for the four disjunction nodes of the Antirrhineae, but also four Old World-to-New World connections, with no colonization events in the opposite direction. In the ancestral range reconstruction using S-DIVA, Old World to New World dispersal events were strongly supported for the *Cymbalaria*, *Galvezia*, *Linaria* and *Sairocarpus* lineages (Fig. 2). DEC results were congruent with an Old World-to-New World pattern for the four dispersal events (Fig. 3).

Fig. 3. Biogeographic reconstruction based on dispersal-extinction-cladogenesis modelling implemented in Lagrange. The maximum clade credibility tree produced by relaxed molecular-clock analysis of *ndh*F sequences of Antirrhineae is shown, after pruning outgroup taxa except for *Lafuentea*.



Temporal congruence

Considering that the four colonization events were found to occur after the last land connection between the Old and the New Worlds (as shown by the Bayesian dating and biogeographic reconstructions), we interpreted four independent long-distance dispersal events. Therefore, a dispersal-mediated allopatry scenario, in which cladogenesis immediately follows dispersal, was considered reasonable for the Antirrhineae, and the timing of colonization events was estimated as coincident with the timing of divergence between Old World and New World lineages. By comparing the posterior distributions of the four TMRCAs (Fig. 2; Table 4), we were able to assess the temporal congruence of the four dispersal events. High PPs for the Miocene were obtained in the *Sairocarpus* (0.99), *Galvezia* (0.97) and *Linaria* (0.93) lineages (Table 4). For the *Cymbalaria* lineage, PP was primarily distributed in the Miocene (0.70), followed by the Oligocene (0.29). Table 5 shows the probabilities of temporal congruence, i.e. the PPs that two, three or four TMCRAs occurred within the bounds of the same geologic epoch. The Miocene was the only epoch which yielded significant probabilities, including a certain probability (PP = 0.62) of temporal congruence of the four lineages taken together. Interestingly, a medium-to-high probability value (PP = 0.89) was retrieved when considering only three lineages (*Galvezia*, *Linaria*, *Sairocarpus*). Therefore, we interpreted that optimal conditions for migration of the Antirrhineae occurred in the Miocene.

Table 4. Posterior probabilities (PP) of occurrence of the most recent common ancestors of Old-New World Antirrhineae lineages within the bounds of six geologic epochs (Eocene-Holocene). *, 0.50 < PP < 0.75; **, 0.75 < PP < 0.90; ***, 0.90 < PP.

Lineage	**Eocene** (55.8-33.9 Ma)	**Oligocene** (33.9-23.03 Ma)	**Miocene** (23.03-5.332 Ma)	**Pliocene** (5.332-5.588 Ma)	**Pleistocene** (2.588-0.0117 Ma)	**Holocene** (0.0117-0 Ma)
Cymbalaria	0.003	0.293	**0.703***	0.000	0.000	0.000
Galvezia	0.000	0.018	**0.969*****	0.013	0.000	0.000
Linaria	0.000	0.001	**0.927*****	0.072	0.001	0.000
Sairocarpus	0.000	0.001	**0.991*****	0.008	0.000	0.000

Table 5. Posterior probabilities (PP) of temporal congruence of two or more Old-New World lineage divergences in six geologic epochs (Eocene-Holocene). *, 0.50 < PP < 0.75; **, 0.75 < PP < 0.90; ***, 0.90 < PP. 1: *Cymbalaria* lineage; 2: *Galvezia* lineage; 3: *Linaria* lineage; 4: *Sairocarpus* lineage.

Lineages	Eocene (55.8-33.9 Ma)	Oligocene (33.9-23.03 Ma)	Miocene (23.03-5.332 Ma)	Pliocene (5.332-5.588 Ma)	Pleistocene (2.588-0.0117)	Holocene (0.0117-0 Ma)
1,2	0.0000	0.0112	**0.6868***	0.0000	0.0000	0.0000
1,3	0.0000	0.0002	**0.6441***	0.0000	0.0000	0.0000
1,4	0.0000	0.0006	**0.6958***	0.0000	0.0000	0.0000
2,3	0.0000	0.0000	**0.8982****	0.0019	0.0000	0.0000
2,4	0.0000	0.0001	**0.9605*****	0.0001	0.0000	0.0000
3,4	0.0000	0.0000	**0.9190*****	0.0007	0.0000	0.0000
1,2,3	0.0000	0.0000	**0.6293***	0.0000	0.0000	0.0000
1,2,4	0.0000	0.0001	**0.6792***	0.0000	0.0000	0.0000
1,3,4	0.0000	0.0000	**0.6372***	0.0000	0.0000	0.0000
2,3,4	0.0000	0.0000	**0.8904****	0.0000	0.0000	0.0000
1,2,3,4	0.0000	0.0000	**0.6225***	0.0000	0.0000	0.0000

Discussion

Synchronous colonization of four snapdragon lineages

The dating analysis herein presented supports that lineage differentiation of Antirrhineae postdated the Eocene, and therefore occurred when a considerable water barrier (the Atlantic Ocean) was present between the Old and the New Worlds (Tiffney & Manchester, 2001). Therefore, we failed to find evidence for vicariance processes as analyzed by lineage divergence times of Old and New World Antirrhineae. Such a biogeographic scenario, with marine barriers, long-distance dispersal and allopatric differentiation has been referred to as dispersal-mediated allopatry (Clark *et al.*, 2008), in which divergence between lineages present in both areas is effectively instantaneous following dispersal (Renner, 2004; Ree *et al.*, 2005). Neither DEC modelling nor S-DIVA analysis include direct inference of dispersal-mediated allopatry, as these analyses model dispersal as happening along branches of the phylogenetic tree (Buerki *et al.*, 2011). Nevertheless, as pointed out by Clark *et al.* (2008), dispersal-mediated allopatry is reconstructed as dispersal followed by vicariance in DIVA analyses, as was found in Antirrhineae (Fig. 1). Given that the sister genus *Lafuentea* and the basal-most lineage of Antirrhineae (the *Anarrhinum-Kickxia* lineage) are currently present in the Old World, our biogeographic reconstructions strongly supported four Old-to-New World colonization events

coupled with allopatric differentiation during the Miocene (Figs. 2, 3). To our knowledge, this is the first time that biogeographic analyses support a synchronous process of colonization within a single plant group, and this agrees with previous predictions for the BCH (Vargas *et al.*, 2004; Wen & Ickert-Bond, 2009).

The BCH revisited

At least four conditions are needed to reliably support the BCH: (i) similar distribution of closely-related taxa in two comparable territories, i.e. lineage disjunctions; (ii) common ancestry of plants involved in the disjunction, as previously hypothesized for the Madrean-Tethyan flora (including the Antirrhineae) based on taxonomy; (iii) identification of sister relationship patterns, e.g. sister lineages currently forming part of the Mediterranean and Californian floras; and (iv) sharing of the same window of colonization in a temporal scale (Cunningham & Collins, 1994; Donoghue & Moore, 2003). As more angiosperms displaying the same biogeographic pattern are found, i.e. multiple lineages showing the same disjunction that occurred in the same period of time, greater support is given to connections between Tertiary relict elements (Donoghue *et al.*, 2001; Xiang & Soltis, 2001; Wen & Ickert-Bond, 2009). The BCH is dependent on the length of each geological period as largely defined by biological events. Therefore, the likelihood of higher or lower biogeographic congruence is tightly related to the window of opportunity offered by the lag span of time of each geologic period.

The above four-fold approach allows for explicitly testing the BCH within a single plant group by using lineage divergence estimates. The BCH appears to be difficult to document within a single plant group of a few lineages; if so, it is interpreted as a patent signal of sharing similar ecological conditions for the two disjunct areas. Our analyses clearly detected biogeographic congruence in the Miocene for four Antirrhineae disjunctions. Miocene connections have also been estimated in other plant lineages included in the Madrean-Tethyan flora (Vargas *et al.*, in prep.; see also Kadereit & Baldwin, 2012), including Cistaceae (Guzmán & Vargas, 2009), Geraniaceae (Fiz-Palacios *et al.*, 2010) and *Cercis* (Davis *et al.*, 2002). Similarly, a review of 33 dated phylogenies of temperate vascular plants upheld the view that eastern Asia and eastern North America were connected by migrations at different times, but mostly in the

Miocene (Donoghue & Smith, 2004). The Miocene was presumably a period of plant expansion through northern corridors in which similar habitat conditions favoured inter-continental colonization (Grímsson *et al.*, 2007). The question remains as to whether shared ecological conditions in the same geological period have been determinant factors accounting for floristic links that conserve similar ecological niches (Tiffney & Manchester, 2001; Smith & Donoghue, 2010). In this regard, it is intriguing the fact that the Californian-Mediterranean disjunction of Antirrhineae and other plant lineages may have originated in an epoch (Miocene) earlier than the establishment of Mediterranean climates on both regions. The Mediterranean climates appear to have been established in the Miocene-Pliocene (7-4 Ma; Millar, 2012) in California, and in the Pliocene (c. 3 Ma; Suc, 1984) in the Mediterranean basin, when Miocene connections had already taken place (Fig. 2). Nevertheless, Mediterranean plants are considered to be dry-adapted species that may belong in lineages with earlier adaptations to summer drying (Millar, 2012). For instance, a dry-tolerant ancestor of Mediterranean-western American lineages has been proposed for the Miocene (7-17 Ma) for *Lonicera*, followed by adaptation to Mediterranean niches (Smith & Donoghue, 2010).

Ancient floras, such as the Madrean-Tethyan, may have left a testable signature in current lineages. Comparative analyses of multiple angiosperm groups may furnish additional information to test the BCH in this and other disjunctions historically proposed by biogeographers (Humboldt, 1817; Darwin, 1859; Raven & Axelrod, 1978), and this may then reflect similar ecological conditions shared by two or more territories over time (Edwards *et al.*, 2007).

Acknowledgements

This research was supported by the Spanish Ministry of Science and Innovation through project CGL2009-10031, by a FPU fellowship (AP2007-01841; Spanish Ministry of Education) to MF-M and by a Marie Curie IEF fellowship “BIRDISLAND” to LMV.

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Apéndice 2

Análisis filogenético del género *Linaria* basado en secuencias del ADN plastidial

Este trabajo se ha desarrollado en colaboración con José Luis Blanco Pastor.

Manuscrito inédito.

Introducción

En el Capítulo 2 de la presente memoria se evaluaron por primera vez las relaciones filogenéticas a gran escala dentro del género *Linaria* mediante el análisis de secuencias del ADN ribosómico nuclear (región ITS). Asimismo, varios estudios ya publicados, o en proceso de publicación, han explorado las relaciones filogenéticas de linajes concretos de *Linaria*, empleando para ello tanto secuencias del ADN nuclear como del ADN plastidial (Sección *Versicolores*: Capítulos 3 y 4, Fernández-Mazuecos & Vargas, 2011; Sección *Supinae*: Blanco-Pastor *et al.*, 2012, Blanco-Pastor & Vargas, en preparación). Sin embargo, no hay disponible aún un análisis filogenético basado en secuencias plastidiales con un muestreo amplio de todo el género. En este anexo presentamos un análisis filogenético de una muestra representativa de especies de todas las secciones de *Linaria*, basado en secuencias de ADN de dos regiones plastidiales. Se discuten las implicaciones sistemáticas de la filogenia obtenida en relación al resto de análisis filogenéticos de *Linaria* previamente realizados.

Material y métodos

Estrategia de muestreo y secuenciación de ADN

Se muestrearon las mismas 96 especies de *Linaria* (incluidas dos especies americanas incluidas en *Nuttallanthus* por Sutton, 1988) secuenciadas en el Capítulo 2 (véase Tabla 3 del Capítulo 2). Se seleccionaron dos regiones del genoma plastidial que previamente habían sido útiles para la reconstrucción filogenética en el género *Linaria*: *rpl*32-*trn*L^{UAG} y *trn*S-*trn*G (Fernández-Mazuecos & Vargas, 2011; Blanco-Pastor *et al.*, 2012; Capítulo 4). Para la extracción de ADN y la secuenciación de las dos regiones se siguieron los protocolos descritos por Fernández-Mazuecos & Vargas (2011; Capítulo 3). Como grupo externo, se seleccionaron una especie del género *Chaenorhinum* y otra de *Antirrhinum*, teniendo en cuenta análisis filogenéticos anteriores (Vargas *et al.*, 2004; Fernández-Mazuecos & Vargas, 2011).

Análisis filogenéticos

Las secuencias se alinearon utilizando el programa MAFFT (Katoh *et al.*, 2002). Tras efectuar ajustes adicionales a mano, las dos regiones (*rpl*32-*trn*L^{UAG} y *trn*S-*trn*G) se concatenaron en una única matriz. Se efectuaron análisis filogenéticos mediante inferencia bayesiana (IB), máxima verosimilitud (MV) y máxima parsimonia (MP) (Goloboff *et al.*, 2003; Guindon & Gascuel, 2003; Ronquist & Huelsenbeck, 2003). En los tres casos, se emplearon los programas informáticos y métodos descritos en Fernández-Mazuecos & Vargas (2011; Capítulo 3).

Resultados

Las características de las dos regiones plastidiales secuenciadas se resumen en la Tabla 1. La matriz alineada tuvo una longitud total de 1461 pb. No se pudieron obtener secuencias de dos de las especies muestreadas (*L. japonica* y *L. latifolia*). Los tres análisis filogenéticos dieron árboles congruentes, aunque se obtuvo una mayor resolución en el análisis bayesiano (Fig. 1). Se recuperaron como monofiléticas las secciones *Versicolores* (probabilidad posterior bayesiana, PP = 1; *bootstrap* de MV, BS-MV = 94%; *bootstrap* de MP, BS-MP = 67%), *Pelisserianae* (PP = 1; BS-MV = 99%; BS-MP = 96%), *Macrocentrum* (PP = 1; BS-MV = 100%; BS-MP = 99%) y *Lectoplectron* (PP = 1; BS-MV = 100%; BS-MP = 100%). Las tres últimas formaron un clado bien apoyado en el análisis bayesiano (PP = 0.95), pero no en el resto de análisis. Otro gran clado, sólo bien apoyado por el análisis bayesiano (PP = 0.95), estuvo formado por las especies de las secciones *Diffusae*, *Linaria*, *Speciosae* y *Supinae*, todas las cuales resultaron como polifiléticas. La sect. *Diffusae* quedó dividida en tres partes (Fig. 1): *L. hirta*, el grupo de *L. reflexa* (PP = 1;

Tabla 1. Características de las dos regiones del ADN plastidial empleadas en los análisis filogenéticos.

	***rpl*32-*trn*L^{UAG}**	**trn*S*-trn*G***
Longitud alineada (pb)	857	604
Rango de longitudes sin *gaps*	501-674	443-567
% identidad por pares	90.2	92.7
Caracteres variables	255	190
Caracteres informativos de parsimonia	158	105
% medio de contenido en G+C	19.5	26.9
Modelo de sustitución	GTR+G	GTR+G

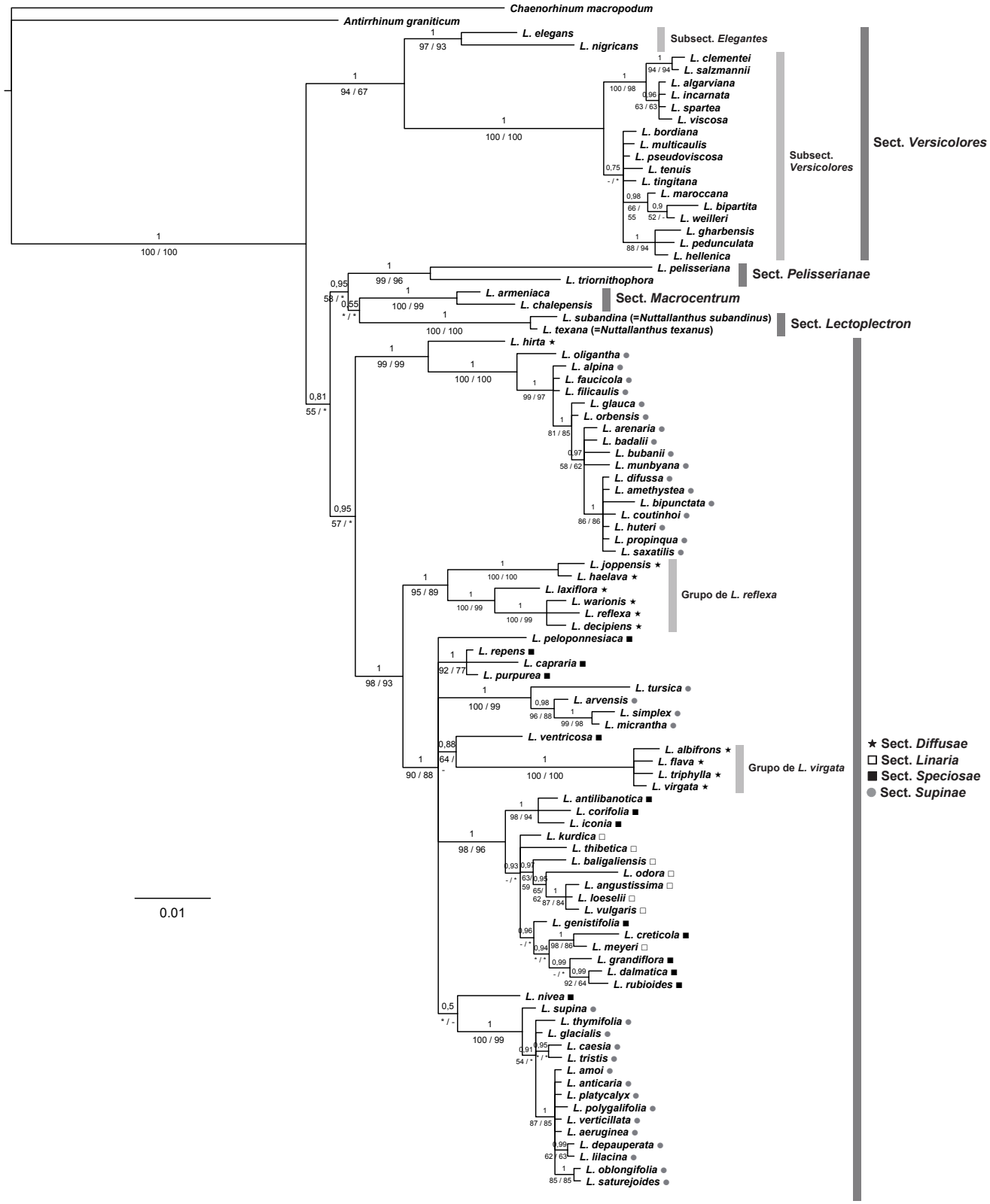
BS-MV = 95%; BS-MP = 89%) y el grupo de *L. virgata* (PP = 1; BS-MV = 100%; BS-MP = 100%). La sect. *Supinae* quedó dividida en tres clados bien apoyados e independientes, mientras que las especies de las secciones *Linaria* y *Speciosae* aparecieron intercaladas a lo largo de un clado que también incluyó el grupo de *L. virgata* y uno de los clados de la sect. *Supinae*.

Discusión

El presente análisis filogenético reafirma la mayoría de las implicaciones sistemáticas previamente extraídas del análisis de secuencias nucleares ITS (Capítulo 2). En primer lugar, se confirma la inclusión en el clado de *Linaria* de las especies americanas previamente tratadas como el género *Nuttallanthus* (Sutton, 1988). Los resultados filogenéticos basados en secuencias nucleares (Capítulo 2) y plastidiales (este Apéndice), junto con la ausencia de caracteres morfológicos especialmente distintivos de *Nuttallanthus* frente a *Linaria* (véase el Capítulo 2), aconsejan tratar estas especies americanas como una sección dentro de *Linaria*: la sección *Lectoplectron* Pennell. Además de *Lectoplectron*, se ha confirmado la monofilia de otras tres secciones de *Linaria*, ya sugerida por la filogenia de ITS: *Macrocentrum*, *Pelisserianae* y *Versicolores*. En los cuatro grupos monofiléticos existen caracteres morfológicos sinapomórficos que apoyan su carácter natural, como se ha discutido en el Capítulo 2 (véase también Valdés, 1970; Viano, 1978a, b; Sutton, 1980, 1988).

En el caso de la sect. *Versicolores*, su monofilia ya había sido apoyada por los diversos análisis filogenéticos incluidos en la presente memoria, que incluyeron un muestreo representativo de otras secciones como grupo externo (Capítulos 3 y 4; Fernández-Mazuecos & Vargas, 2011). Se confirma, por tanto, la condición de grupo natural de *Versicolores*, tal y como ya se había propuesto a partir del análisis de caracteres morfológicos (particularmente el estigma bipartito que constituye una sinapomorfía de la sección) (Sutton, 1988). Asimismo, se confirma el carácter natural de las dos subsecciones de *Versicolores* (*Elegantes* y *Versicolores*) y su condición de grupos hermanos, tal y como se ha obtenido de manera recurrente en los distintos análisis incluidos en esta memoria (Capítulos 2, 3 y 4).

La polifilia obtenida para la sección *Supinae* es congruente con la obtenida, utilizando los mismos marcadores plastidiales, en el análisis de Blanco-Pastor *et al.* (2012), basado en un


Chaenorhinum macropodum
Antirrhinum graniticum
L. elegans
L. nigricans
Subsect. Elegantes
L. clementei
L. salzmannii
L. algarviana
L. incarnata
L. spartea
L. viscosa
L. bordiana
L. multicaulis
L. pseudoviscosa
L. tenuis
L. tingitana
L. maroccana
L. bipartita
L. weilleri
L. gharbensis
L. pedunculata
L. hellenica
Subsect. Versicolores
Sect. Versicolores
L. pelisseriana
L. triornithophora
Sect. Pelisserianae
L. armeniaca
L. chalepensis
Sect. Macrocentrum
L. subandina (=Nuttallanthus subandinus)
L. texana (=Nuttallanthus texanus)
Sect. Lectoplectron
L. hirta ★
L. oligantha ●
L. alpina ●
L. faucicola ●
L. filicaulis ●
L. glauca ●
L. orbensis ●
L. arenaria ●
L. badalii ●
L. bubanii ●
L. munbyana ●
L. difussa ●
L. amethystea ●
L. bipunctata ●
L. coutinhoi ●
L. huteri ●
L. propinqua ●
L. saxatilis ●
L. joppensis ★
L. haelava ★
L. laxiflora ★
L. warionis ★
L. reflexa ★
L. decipiens ★
Grupo de L. reflexa
L. peloponnesiaca ■
L. repens ■
L. capraria ■
L. purpurea ■
L. tursica ●
L. arvensis ●
L. simplex ●
L. micrantha ●
L. ventricosa ■
L. albifrons ★
L. flava ★
L. triphylla ★
L. virgata ★
Grupo de L. virgata
L. antilibanotica ■
L. corifolia ■
L. iconia ■
L. kurdica □
L. thibetica □
L. baligaliensis □
L. odora □
L. angustissima □
L. loeselii □
L. vulgaris □
L. genistifolia ■
L. creticola ■
L. meyeri □
L. grandiflora ■
L. dalmatica ■
L. rubioides ■
L. nivea ■
L. supina ●
L. thymifolia ●
L. glacialis ●
L. caesia ●
L. tristis ●
L. amoi ●
L. anticaria ●
L. platycalyx ●
L. polygalifolia ●
L. verticillata ●
L. aeruginea ●
L. depauperata ●
L. lilacina ●
L. oblongifolia ●
L. saturejoides ●
0.01
★ Sect. Diffusae
□ Sect. Linaria
■ Sect. Speciosae
● Sect. Supinae

muestreo más reducido (aunque representativo) del género. Sin embargo, estos autores demostraron, mediante simulaciones del proceso de coalescencia, que la sección *Supinae* es, en realidad, monofilética. La ausencia de monofilia obtenida para distintos marcadores moleculares (incluidos los plastidiales), así como la incongruencia entre marcadores a este nivel, serían debidas, según los resultados de Blanco-Pastor *et al.*, al proceso conocido como "repartición incompleta de linajes" (más conocido por el término en inglés *incomplete lineage sorting*). Los tres clados pertenecientes a la sección *Supinae* obtenidos en nuestro análisis de secuencias plastidiales (Fig. 1) son congruentes con la delimitación de las tres subsecciones morfológicamente bien definidas propuestas por Blanco-Pastor *et al.* (2012): *Arvenses*, *Saxatile* y *Supinae* (véase también Valdés, 1970; Sutton, 1988).

El patrón de polifilia obtenido para la sect. *Diffusae* coincide con el encontrado en la filogenia de ITS (Capítulo 2), con dos grupos naturales filogenéticamente bien definidos (los grupos de *L. reflexa* y *L. virgata*) y una especie aislada (*L. hirta*) afín a especies de la sect. *Supinae*. Por tanto, los resultados basados en secuencias, tanto nucleares como plastidiales, apoyan la propuesta, avanzada en el Capítulo 2, de tratar el grupo de *L. reflexa* como sect. *Diffusae sensu stricto* y el grupo de *L. virgata* como sect. *Minutiflorae* (Bentham, 1846; Valdés, 1970). Esta propuesta se ve también apoyada por los análisis basados en coalescencia de Blanco-Pastor *et al.* (2012). En cambio, las afinidades sistemáticas de *L. hirta* permanecen irresolutas.

Fig. 1. Relaciones filogenéticas entre 94 especies de *Linaria* (incluidas dos especies anteriormente incluidas en *Nuttallanthus*) basadas en el análisis combinado de dos regiones del ADN plastidial (*rpl*32-*trn*L^{UAG} y *trn*S-*trn*G). Se muestra el árbol consenso (obtenido por la regla de la mayoría del 50%) resultante del análisis bayesiano. Los números sobre las ramas son las probabilidades posteriores bayesianas. Los números bajo las ramas son los valores de *bootstrap* de máxima parsimonia / máxima verosimilitud. Un asterisco (*) indica un clado con apoyo *bootstrap* por debajo del 50%, pero presente en el árbol de máxima verosimilitud o el árbol de consenso estricto del análisis de máxima parsimonia. Un guión (-) indica un clado con apoyo *bootstrap* por debajo del 50%, y ausente en el árbol de máxima verosimilitud o el árbol de consenso estricto del análisis de máxima parsimonia. Se indican los linajes discutidos en el texto. La delimitación de las secciones sigue a Sutton (1988), a excepción de la sect. *Lectoplectron*, que sigue a Valdés (1970).

Las afinidades de las especies de las secciones *Linaria* y *Speciosae* claramente requieren un estudio más profundo. En la filogenia de ITS (Capítulo 2), ambas secciones aparecieron entremezcladas en un clado que también incluyó el grupo de *L. virgata*. En la filogenia plastidial (Fig. 1), aparecen igualmente asociadas al grupo de *L. virgata*, pero también a dos de los linajes de la sect. *Supinae*, lo mismo que en la filogenia plastidial de Blanco-Pastor *et al.* (2012). En el árbol de especies basado en coalescencia de estos últimos autores (realizado a partir de secuencias plastidiales y nucleares), las nueve especies incluidas de las secciones *Linaria* y *Speciosae* formaron un clado junto con el grupo de *L. virgata* (Fig. 1). Esto sugiere que el patrón de aparente polifilia del grupo *Linaria-Speciosae* en el árbol plastidial podría deberse, en buena parte, a la repartición incompleta de linajes. En cualquier caso, la baja resolución obtenida en este grupo por Blanco-Pastor *et al.* (2012), así como el escaso muestreo de la sect. *Linaria* del que adolecen todas las filogenias realizadas hasta el momento, hacen que la naturalidad de las secciones *Linaria* y *Speciosae*, así como sus relaciones con el grupo de *L. virgata*, requieran una mayor investigación.

La presente filogenia basada en secuencias plastidiales, junto con la basada en secuencias nucleares ITS (Capítulo 2) y los análisis basados en coalescencia de Blanco-Pastor *et al.* (2012), viene a confirmar el carácter artificial de la división del género *Linaria* en dos grupos sobre la base de la presencia o ausencia de ala en las semillas (Viano, 1978b). A lo largo de la evolución del género, parecen haber tenido lugar múltiples cambios entre semillas aladas y no aladas, tal y como se ha discutido en el Capítulo 2. El efecto que pudieran haber tenido estos cambios en los patrones biogeográficos y de colonización del género (Willson & Traveset, 2000) está aún por explorar.

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Apéndice 3

Análisis biométricos y filogenéticos apoyan tres especies crípticas dentro del complejo ibero-norteafricano *Linaria incarnata*

Biometric and phylogenetic analyses support two cryptic species in the Ibero-North African *Linaria incarnata* complex

Este trabajo se ha desarrollado en colaboración con Beatriz Vigalondo (Universidad Autónoma de Madrid) y Llorenç Sáez (Universitat Autònoma de Barcelona).

Manuscrito actualmente en preparación:

Vigalondo B, Fernández-Mazuecos M, Vargas P, Sáez L. Biometric and phylogenetic analyses support two cryptic species in the Ibero-North African *Linaria incarnata* complex. En preparación.

Abstract

The toadflax *Linaria incarnata* (Vent.) Spreng. has been treated as a widely-distributed Ibero-North African species in the last taxonomic treatments of the genus *Linaria*. However, morphological and phylogenetic disparity between populations of this taxon has been previoulsy suggested. Here we present new morphological and phylogenetic evidence for disintegration of the *L. incarnata* complex into three distinct species: *L. incarnata s.s.* in the Western Iberian Peninsula; *L. mamorensis* Mazuecos, Vigalondo & L.Sáez sp. nov. in Morocco; and *L. onubensis* Pau in southwestern Spain. Given the poor morphological differentiation between these taxa (which can be regarded as cryptic species), yet their distinct phylogenetic positions, convergent evolution accounts for the independent origins of *L. incarnata*-type morphologies in the course of the evolution of *Linaria* sect. *Versicolores*.

Introduction

Linaria Mill. (toadflaxes) is the largest genus of the tribe Antirrhineae (Sutton, 1988). It is distributed throughout the Palearctic region and America, and has its main centre of species diversity in the Mediterranean region (southern Europe, northern Africa and southwestern Asia) (Valdés, 1970; Sutton, 1988; Chapter 2). Toadflax species are annual, biennial or perennial herbs, with heteromorphic stems. Flowers are zygomorphic, with a personate, spurred corolla. They are arranged in terminal, bracteate racemes, spikes or panicles, usually with accrescent pedicels. Fruits are capsules that contain a variable number (three to >120) of seeds. Seed morphology is remarkably diverse across the genus, which has been divided in two main groups according to the presence or absence of a marginal encircling wing in seeds (Valdés, 1970; Viano, 1978c; Sutton, 1988). Sutton (1988) provided the last taxonomic revision of the whole genus, which recognized 150 species. Recently, Sáez & Bernal (2009) presented a detailed review for the Iberian Peninsula, which included 90 taxa at specific or subspecific level. The genus (including the four American species formerly circumscribed as *Nuttallanthus* D.A.Sutton) has been found to be monophyletic based on phylogenetic analyses of nuclear (Chapter 2) and plastid (Appendix 2) DNA regions.

Remarkable taxonomic complexity is found in *Linaria*, as shown by the conflicting taxonomic treatments of many taxa and species groups found in the bibliography (Valdés, 1970; Viano, 1978b, c; Sutton, 1988; Sáez & Bernal, 2009), and the ongoing taxonomic revisions and rearrangements of different groups (Segarra & Mateu, 2001b; Sáez *et al.*, 2004; Sáez & Crespo, 2005; Sáez *et al.*, 2008). Indeed, morphological traits separating closely related taxa are frequently scanty, probably due to recent speciation (Valdés, 1970; Viano, 1978b; Blanco-Pastor *et al.*, 2012; Chapter 3; Chapter 4). As in other Antirrhineae genera, seed morphology and seed-coat surface sculpturing are usually relevant characters to discriminate *Linaria* species (Valdés, 1970; Elisens & Tomb, 1983; Elisens, 1985; Juan *et al.*, 1999, 2000; Segarra & Mateu, 2001a), together with other morphological traits, such as habit, indumentum, corolla shape and colour, etc. (Viano, 1969; Valdés, 1970; Sutton, 1988; Sáez & Bernal, 2009).

Section *Versicolores* (Benth.) Wettst. is an assemblage of c. 22 species of *Linaria* with wingless (reniform or trigonous) seeds mainly distributed in the western Mediterranean region (Viano, 1978b, c; Sutton, 1988). This is one of the most distinctive subdivisions of *Linaria*, due to the

divided style with discrete stigmatic areas, a feature not found elsewhere in the genus (Sutton, 1988). Two subsections are currently recognized within section *Versicolores* (Sutton, 1988): subsect. *Versicolores* (c. 20 species with a conspicuously bifid style), and subsect. *Elegantes* (two species, *L. elegans* and *L. nigricans*, with an emarginated stigma). According to the most recent taxonomic revisions (Viano, 1978b, c; Sutton, 1988; Sáez & Bernal, 2009) ten species are recorded for the Iberian Peninsula, mainly distributed throughout the southern regions. Six of them are Iberian endemics: *L. algarviana*, *L. viscosa*, *L. salzmannii*, *L. clementei*, *L. elegans* and *L. nigricans*; one species is shared with southern France: *L. spartea*; and three are shared with northern Africa: *L. incarnata*, *L. pedunculata* and *L. gharbensis*. Recent phylogenetic analyses based on plastid (Fernández-Mazuecos & Vargas, 2011; Chapter 3; Appendix 2) and nuclear (Chapter 2) DNA sequences supported section *Versicolores* as a natural group, with subsections *Versicolores* and *Elegantes* also supported as monophyletic groups that are sister to each other.

Linaria incarnata (Vent.) Spreng. belongs to a group of species of subsect. *Versicolores* characterized by a particular corolla shape with a narrow tube and a poorly developed palate that scarcely occludes the tube (Viano, 1969). The current circumscription of this species dates back to Viano (1969), who separated it from the similar *L. bipartita* based on the densely glandulous-pubescent inflorescence of *L. incarnata* (as opposed to the entirely glabrous *L. bipartita*), and from *L. elegans* based on the bifid style of *L. incarnata* (as opposed to the simple style with emarginated stigma of *L. elegans*). As defined by this author, *L. incarnata* is an Ibero-North African species distributed in the Atlantic areas of the two sides of the strait of Gibraltar: Portugal, western Spain and western Morocco. This circumscription has been accepted in all subsequent taxonomic revisions and floras of the Iberian Peninsula and Morocco (Viano, 1978b; Valdés, 1987; Sutton, 1988; Valdés *et al.*, 2002; Gómiz, 2004; Sáez & Bernal, 2009). Sutton (1988), although following Viano's concept of *L. incarnata*, was the first to highlight some morphological differences between Iberian and north African specimens, including the shape of bracts, calyx-lobes and stigma. This author went as far as to suggest that "further investigation may reveal that the north African and European specimens form distinct taxa". However, no further morphological analyses have been conducted since then. A recently-published phylogenetic analysis of sect. *Versicolores* based on plastid DNA (cpDNA) (Fernández-Mazuecos & Vargas, 2011; Chapter 3) supported the polyphyly of *L. incarnata*, as sequenced

individuals from the Iberian Peninsula and northern Africa were revealed as belonging to largely Iberian and north African clades respectively.

In a preliminary study of *L. incarnata*, we observed that some herbarium specimens from southwestern Spain (Huelva province, western Andalusia) did not to exhibit the dense inflorescence indumentum that characterizes the species. In addition, some inconsistent traits were found in the descriptions of *L. incarnata* included in the regional flora of Western Andalusia (Valdés, 1987) and Flora iberica (Sáez & Bernal, 2009). Valdés' (1987) description was similar to the conflictive specimens, and also resembled that of *L. onubensis* Pau, a species described from collections from the same area (Pau, 1933). Based on Pau's description, Sutton (1988) suggested that *L. onubensis* may correspond to *L. elegans*, although this author did not examine any material. The clearly bifid style of the type material (preserved in the MA herbarium), however, clearly places these plants in subsect. *Versicolores* (and not in subsect. *Elegantes*, to which *L. elegans* belongs), which led Valdés (1987) to accept *L. onubensis* as a synonym of *L. incarnata*.

Further research is therefore needed in order to clarify the taxonomic status of different populations of the *L. incarnata* complex. Integration of detailed morphological and DNA sequence-based phylogenetic analyses help accomplish a well-supported systematic revision, particularly when poorly-differentiated complexes, potentially constituted by cryptic species, are involved (Dayrat, 2005; Bickford *et al.*, 2007; De Queiroz, 2007; Wiens, 2007). Here we present an integrative assessment of the *L. incarnata* complex (henceforth *L. incarnata s.l.*) based on the morphological analysis of vegetative, floral and fruit traits, scanning electronic microscopy evaluation of seed shape and seed-coat surface sculpturing, and phylogenetic analyses (based on nuclear and plastid DNA sequences) performed in the context of an ongoing evolutionary study of sect. *Versicolores* (Fernández-Mazuecos & Vargas, 2011; Chapters 3-5). New information on habitat and distribution is also provided. The ultimate goal was to provide a well-supported systematic reassessment and to interpret evolutionary patterns of the *L. incarnata* complex.

Materials and methods

Morphological analysis

Specimens of *L. incarnata s.l.* were obtained from five herbaria (ABH, MA, SALA, SEV and RNG), and from the authors' collections (see Appendix S1). Voucher specimens of newly-sampled localities were deposited in the herbaria of the Department of Botany at the Universitat Autònoma de Barcelona (BCB) and the Real Jardín Botánico in Madrid (MA), Spain. Although the main objective was to investigate the taxonomic status of *L. incarnata* populations, we also obtained morphometric data from four other species and subspecies of sect. *Versicolores* potentially related to *L. incarnata s.l.*: *L. algarviana*, *L. spartea*, *L. viscosa* subsp. *viscosa* and *L. viscosa* subsp. *spicata*.

Morphological characters were selected following Sáez & Bernal (2009), Sutton (1988) and our own observations of field and herbarium specimens. The selected characters included 21 quantitative and seven qualitative variables (Tables 1, 2). A total of 148 specimens were examined for all variables, except for four qualitative variables for which a more limited sampling was performed: seed shape, seed-coat sculpturing, periclinal testa cell ornamentation and trichome type (only evaluated for *L. incarnata s.l.* populations).

Measurements of the vegetative and floral parts were carried out under a stereomicroscope ZEISS Stemi DV4 using rulers and an electronic digital caliper. Trichomes of *L. incarnata s.l.* populations were examined under an AxioScope-ZEISS light microscope at 100–600x magnification. Dry mature seeds were analyzed with scanning electron microscopy (SEM). They were glued directly to aluminum stubs, coated with 40-50 nm gold and examined with a HITACHI 2300-S at 15 kV. Terminology of trichome types and indumentum density followed Payne (1978) and Segarra & Mateu (2001a), and that of seed shape and seed-coat surface sculpturing followed Sutton (1988).

Descriptive statistics (means, standard deviations and ranges) were computed for quantitative characters. Then, an ordination analysis was performed for all characters. Given the presence of quantitative and qualitative morphological characters, principal coordinate analysis (PCoA) was conducted using Gower's coefficient of similarity for mixed data (Gower, 1971), in order

to represent the morphological affinities among specimens and species. Multivariate analysis of variance (MANOVA) was also performed to assess the discriminant value of the quantitative characters for separating previously recognized *Linaria* taxa and the three groups of *L. incarnata s.l.* populations previously suggested by taxonomists (Pau, 1933; Sutton, 1988): (1) Iberian populations excluding *L. onubensis* (henceforth *L. incarnata s.s.*); (2) Moroccan populations; and (3) populations from southwestern Spain named as *L. onubensis* by Pau (1933) (henceforth Huelva populations). A Tukey test was used as a post-hoc analysis to determine the importance of each character in the differentiation of all taxa and *L. incarnata s.l.* population groups. The most discriminant characters for *L. incarnata s.l.* population groups were summarized in the form of box plot graphs.

Two different data sets were used for PCoA and MANOVA analyses: one included specimens from all studied taxa of sect. *Versicolores*, and the other only included specimens of *L. incarnata s.l.* Specimens with missing values were excluded from both data sets, as were variables with few entries (including qualitative variables seed shape, seed-coat sculpturing and trichome type). This led to the exclusion of *L. algarviana* from these analyses, due to the presence of only three specimens with complete data. We used the basic packages of R v.2.15.0 (R Development Core Team, 2012) and the packages *ape* v.3.0-3 (Paradis *et al.*, 2004) and *vegan* v.2.0-3 (Oksanen *et al.*, 2011) to construct the PCoA. The remaining statistical analyses were performed using SPSS v.15 (SPSS, Chicago, Illinois, USA).

Phylogenetic analysis

For molecular phylogenetic analyses, ten individuals of *L. incarnata s.l.* were sampled to cover the entire distribution of the complex: eight individuals from the Iberian Peninsula (including five of *L. incarnata s.s.* and three from Huelva populations) and two individuals from northwestern Africa. Plant material was collected in the field and dried in silica gel or obtained from herbarium collections (RNG) (Table S1). Specimens from the same populations were also included in morphological analyses (see above).

Procedures used for DNA extraction, amplification and sequencing mostly followed Fernández-Mazuecos & Vargas (2011). One nuclear (ITS) and three plastid (*rpl*32-*trn*L^{UAG}, *trn*S-*trn*G,

Table 1. Qualitative and quantitative characters for *L. incarnata s.l.* and relatives. All measurements are in mm, except for stem length (cm) and pedicele angle (degrees). Number (n) of specimens examined for each species is given after taxon names.

	Character	*L. incarnata s.s.* n = 20	*L. incarnata s.l.*, Morocco (= *L. mamorensis*) n = 19	*L.incarnata s.l.*, SW Spain (= *L. onubensis*) n = 23	*L. algarviana* n = 10	*L. spartea* n = 22	*L.viscosa* subsp. *viscosa* n = 20	*L.viscosa* subsp. *spicata* n = 10
Vegetative parts	Stem length	18.7 - 56	14 - 41	18.8 - 59.4	9.6 - 16.8	17.3 - 72.3	8.3 - 19.8	22.7 - 46.1
	Leaf length on sterile shoots	3 - 8.29	2.75 - 11	1.9 - 10.15	4.69 - 10.31	4.82 - 14.94	2.9 - 5.66	2 - 10.57
	Leaf width on sterile shoots	0.5 - 2.1	0.59 - 1.67	0.55 - 1.99	1.52 - 3.13	0.77 - 3.57	0.74 - 2.31	0.72 - 2.15
	Leaf length on fertile shoots	5.5 - 18.68	8 - 32.01	5.87 - 31.39	6.15 - 13.66	8.09 - 37.87	4.21 - 19.36	9.47 - 41.5
	Leaf width on fertile shoots	0.48 - 1.5	0.54 - 1.6	0.24 - 1.16	0.61 - 1.88	0.61 - 1.62	0.47 - 1.1	0.28 - 1.26
Inflorescence	Flower pedicel length	5.3 - 13.5	5 - 13	2.85 - 9	3.34 - 8.44	4.16 - 14.85	4.07 - 7.15	3.89 - 17.69
	Fruit pedicel length	7.27 - 16	8 - 13	5.33 - 12.06	6.36 - 10.17	6.69 - 15.15	4.4 - 8.39	6.12 - 23.09
	Pedicel angle	20 - 47.5	25 - 47.5	35 - 60	35 - 55	0 - 30	5 - 30	5 - 45
	Adnate pedicel	No	No	No	No	Yes	Yes	Variable
	Indumentum density	Densely hairy	Sparsely hairy	Glabrous to sparsely hairy	Sparsely hairy	Glabrous to sparsely hairy	Densely hairy	Sparsely to densely hairy
	Trichome length	0.1 - 1	0.25 - 0.66	0.1 - 0.32	0.1 - 0.34	0.16 - 0.72	0.26 - 0.67	0 - 0.33
	Bract length	2.65 - 6	1.8 - 3.2	1.36 - 5.46	2.24 - 3.44	3.32 - 6.68	1.4 - 3.99	1.74 - 6.05
Flower	Corolla colour	Blue-violet	Blue-violet	Blue-violet	Blue-violet	Yellow	Yellow	Yellow
	Flower calyx-lobe length	1.24 - 4.13	2.15 - 2.98	1.71 - 3	2.96 - 4.51	2.27 - 6.42	2.06 - 3.8	2.49 - 4.74
	Flower calyx-lobe width	0.3 - 1.05	0.51 - 0.875	0.4 - 0.925	0.72 - 1.09	0.53 - 1.5	0.42 - 0.79	0.77 - 1.28
	Corolla length	13.1 - 22.5	15.87 - 23	10.62 - 18.5	18.43 - 24.89	20.36 - 31.18	13.9 - 23.8	14.74 - 27.89
	Spur length	7 - 13.4	8.88 - 13.12	5.92 - 9.78	6.94 - 13.23	8.15 - 14.6	5.43 - 10.26	7.29 - 15.45
	Spur width	0.6 - 1.5	0.77 - 1.41	0.58 - 1.26	1.22 - 2.22	0.8 - 2.05	0.69 - 1.51	0.75 - 1.71
	Adaxial lip length	4.3 - 9	5.08 - 7.3	3.18 - 6.88	5.6 - 7.67	4.77 - 9.8	4.98 - 9.59	4.53 - 8.4
	Adaxial lip sinus length	2.83 - 6	1.77 - 5	0.95 - 4.75	1.1 - 3.04	1.84 - 4.64	0.89 - 5.76	0.91 - 3.66
	Abaxial lip sinus length	1.23 - 2.99	1.11 - 3.5	0.87 - 2.48	0.74 - 1.2	0.8 - 1.92	0.44 - 1.39	0.48 - 1.98
Fruit	Fruit calyx-lobe length	2.5 - 5.07	2.67 - 4.14	1.99 - 4.29	3.14 - 4.1	3.86 - 6.2	2.5 - 3.49	2.73 - 4.7
	Fruit calyx-lobe width	0.5 - 1.3	0.55 - 1.1	0.45 - 1	1.02 - 1.15	0.89 - 2.29	0.65 - 0.99	0.77 - 1.32
	Fruit length	1.63 - 4.25	3.22 - 4	2.19 - 3.58	3.57 - 4.14	3.1 - 5.9	2.33 - 4.1	2.86 - 4.61
Seed	Seed shape	Subtrigonous	Reniform	Reniform	Pyriform-triquetrous	Trigonous-reniform	Reniform	Reniform
	Seed-coat sculpturing	Ruminate-alveolate, ridges rounded	Transversely ridged, ridges rounded to subacute	Transversely ridged, ridges rounded	Transversely ridged, ridges rounded	Transversely ridged, ridges rounded	Transversely ridged, ridges rounded	Transversely ridged, ridges rounded to subacute
	Periclinal testa cell ornamentation	Marginal papilla	Marginal papilla	Marginal and medial papilla	Marginal and medial papilla	Marginal papilla	Marginal papilla	Marginal papilla

Table 2. Quantitative and qualitative characters included in the morphological study and P values resulting from the Tukey test. Notes: * Variables not included in statistical analyses; QN = quantitative variable; QL = qualitative variable; I = *L. incarnata s.s.*; M = *L. incarnata s.l.*, Moroccan populations; O = *L. incarnata s.l.*, southwestern Spain (Huelva) populations.

	Variable	Variable type	Tukey test		
			I - M	I - O	M - O
Vegetative parts	Stem length (cm)	QN	n.s.	n.s.	n.s.
	Leaf length on sterile shoots (mm)	QN	0.013	n.s.	0.002
	Leaf width on sterile shoots (mm)	QN	n.s.	n.s.	n.s.
	Leaf length on fertile shoots (mm)	QN	0.017	0.079	n.s.
	Leaf width on fertile shoots (mm)	QN	n.s.	< 0.001	< 0.001
Inflorescence	Flower pedicel length (mm)	QN	n.s.	< 0.001	0.033
	Fruit pedicel length (mm)	QN	0.009	< 0.001	n.s.
	Pedicel angle (degrees)	QN	n.s.	n.s.	n.s.
	Adnate pedicel [adnate (1), not adnate (2)]	QL	.	.	.
	Indumentum density [glabrous (1), sparsely hairy (2), densely hairy (3)]	QL	.	.	.
	Trichome type*	QL	.	.	.
	Trichome length (mm)	QN	0.001	< 0.001	0.001
	Bract length (mm)	QN	0.001	0.04	n.s.
Flower	Corolla colour [yellow (1), blue-violet (2)]	QL	.	.	.
	Flower calyx-lobe length (mm)	QN	0.018	0.001	n.s.
	Flower calyx-lobe width (mm)	QN	n.s.	< 0.001	< 0.001
	Corolla length (mm)	QN	n.s.	< 0.001	< 0.001
	Spur length (mm)	QN	n.s.	< 0.001	< 0.001
	Spur width (mm)	QN	n.s.	n.s.	n.s.
	Adaxial lip length (mm)	QN	n.s.	0.011	0.001
	Adaxial lip sinus length (mm)	QN	n.s.	< 0.001	< 0.001
	Abaxial lip sinus length (mm)*	QN	.	.	.
Fruit	Fruit calyx-lobe length (mm)	QN	0.016	< 0.001	n.s.
	Fruit calyx-lobe width (mm)	QN	n.s.	0.004	0.019
	Fruit length (mm)	QN	n.s.	0.099	0.012
Seed	Seed shape*	QL	.	.	.
	Seed-coat sculpturing*	QL	.	.	.
	Periclinal testa cell ornamentation*	QL	.	.	.

*ndh*F) regions were used to infer phylogenetic relationships. The nuclear ribosomal internal transcribed spacers (ITS) were sequenced for all ten individuals of *L. incarnata s.l.* using the following PCR conditions: 1 min pretreatment at 94ºC and 30 cycles of 1 min at 94ºC, 1 min at 56ºC and 1 min at 72ºC. We used the external primers 17SE and 26SE (Sun *et al.*, 1994) for amplification, and the internal primers ITS5 (Sang *et al.*, 1995) and ITS4 (White *et al.*, 1990) for sequencing. Additionally, ITS sequences from previous studies were retrieved for 31 individuals representing 27 other species of *Linaria* (including 22 individuals from 18 species of sect. *Versicolores*, and 9 individuals representing the remaining six sections of *Linaria*), one individual of *Antirrhinum* and one of *Chaenorhinum* to be used as the outgroup.

Given that Moroccan populations of *L. incarnata s.l.* were clearly separated from *L. incarnata s.s.* and Huelva populations in a previous cpDNA-based phylogenetic analysis (Fernández-Mazuecos & Vargas, 2011; Chapter 3), here we focused on resolving phylogenetic relationships within the Iberian cpDNA clade of *Linaria* sect. *Versicolores* including *L. incarnata s.s.* and specimens from Huelva (clade II of Fernández-Mazuecos & Vargas, 2011; see Chapter 3). Two regions (*trn*S-*trn*G and a fragment of *ndh*F) were added to the previously published sequences of the *rpl*32-*trn*L^{UAG} spacer. The *trn*K-*mat*K region, used by Fernández-Mazuecos & Vargas (2011), was not further sequenced because it yielded very low nucleotide variation within the clade of interest. The three regions were obtained for all ten individuals of *L. incarnata s.l.* and 16 individuals representing 12 species of *Linaria* sect. *Versicolores*, plus *L. chalepensis* and *L. vulgaris*. PCR and sequencing procedures followed Fernández-Mazuecos & Vargas (2011). The same standard primers (Olmstead & Reeves, 1995; Hamilton, 1999; Shaw *et al.*, 2007) were employed for amplification and sequencing. In the case of *ndh*F, we used primers 972F and 2112R from Olmstead & Sweere (1994) and Olmstead & Reeves (1995) respectively.

All sequences were aligned using MAFFT 6 (Katoh *et al.*, 2002) with default parameters, and further adjustments were made by visual inspection. The three cpDNA regions were concatenated in a single matrix. Phylogenetic analyses were separately performed on the ITS and cpDNA matrices using three methods: Bayesian inference (BI), maximum likelihood (ML), and maximum parsimony (MP). Based on previous phylogenetic evidence (Vargas *et al.*, 2004; Chapter 2), *Chaenorhinum* was employed as the outgroup for the ITS analysis, and *L. chalepensis* for the cpDNA analysis. For BI and ML analyses, the best-fitting substitution models (Table 3)

Table 3. Characteristics of the four DNA regions sequenced for phylogenetic analyses of the *L. incarnata* complex.

	ITS	***rpl*32-*trn*L^{UAG}**	***trn*S-*trn*G**	***ndh*F**	**Combined cpDNA**
Sequences	43	28	28	28	28
Aligned length (bp)	603	730	601	658	1989
Ungapped length range	573-592	700-720	467-582	649-658	1825-1943
Pairwise % identity	92.0	97.1	94.7	98.1	96.7
Variable characters	189	79	57	71	207
Parsimony-informative characters	127	46	16	32	94
Mean % G+C content	58.8	26.7	28.5	27.7	27.6
Substitution model	GTR+G	GTR+G	GTR+G	GTR+I	GTR+I+G

were determined under the Akaike Information Criterion (AIC) in jModelTest 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). BI was performed in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) using two runs with 10 million generations each and a sample frequency of 1000. In the plastid analysis, the three regions were partitioned, and substitution models were unlinked across partitions. Chain convergence was assessed with Tracer 1.4 (Rambaut & Drummond, 2007). Fifty-percent majority rule consensus trees with Bayesian posterior probabilities (PP) of clades were calculated after removing the first 10% generations as burn-in. ML analyses were implemented in PhyML 3.0 (Guindon & Gascuel, 2003; Guindon *et al.*, 2010) using the subtree pruning and regrafting (SPR) branch-swapping algorithm. One-thousand non-parametric bootstrap replicates (ML-BS) were applied. MP analyses were conducted in TNT 1.1 (Goloboff *et al.*, 2003). We used a heuristic search with 10,000 replicates saving two most-parsimonious trees per replicate, followed by a second heuristic search retaining all best trees and using the trees obtained in the previous 10,000 replicates as the starting ones. Bootstrap support (MP-BS) of clades was assessed with 10,000 standard replicates. Given the noticeable incongruence between phylogenetic trees based on ITS and cpDNA sequences (see below), a total-evidence analysis was not performed.

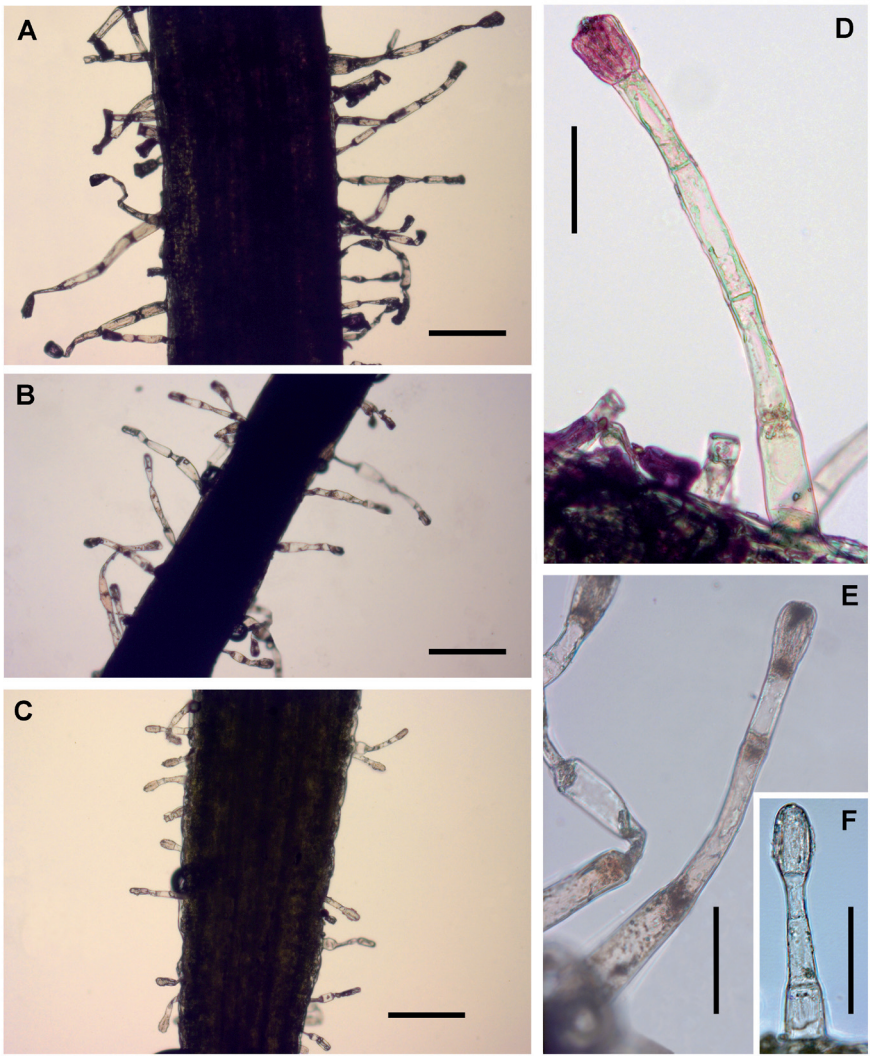


Fig. 1. Light microscope images showing the differences in indumentum density, trichome length and trichome type between *L. incarnata s.s.* (A, D; Morille, Salamanca, ABH 44296), *L. incarnata s.l.* from Morocco (= *L. mamorensis*) (B, E; Kenitra, entre Sidi-Yahya-du-Rharb y Sidi Slimane, SEV 160821), and *L. incarnata s.l.* from southwestern Spain (= *L. onubensis*) (C, F; El Saltillo, Huelva). Scale bars: A-C, 200 µm; D-F, 100 µm.

Results

Taxonomic characters

Indumentum: In most of the studied species, inflorescence axis, pedicels, bracts and calyx lobes were covered by glandular trichomes formed by a gland and a uniseriate stalk. Trichome length was variable, being the shortest in Huelva populations of *L. incarnata s.l.*, and longest in *L. incarnata s.s.* (Table 1; Fig. 1). The number of stalk cells varied from one to eigth, and the

number of cells of the gland ranged from two to eigth. The transverse walls were hialine or light grey in most of the studied species, with the exception of plants from Iberian populations of *L. incarnata s.s.*, which presented transverse walls (and glands) purple to violet. The thickness of the stalk cell wall was also variable within *L. incarnata s.l.*, ranging from 0.9-2.8 μm in Huelva populations to 3.2-7.1 μm in *L. incarnata s.s.* Indumentum density varied considerably among the studied species (Table 1). This feature was useful, together with the inflorescence density, to tell apart two closely related species: *L. spartea* and *L. viscosa*. Density of trichomes was variable amongst the different groups recongnized within *L. incarnata s.l.*: dense in Iberian populations of *L. incarnata s.s.* and sparse (sometimes glabrescent) in African and Huelva populations of *L. incarnata s.l.* (Fig. 1).

Flower characters: In addition to quantitative differences in flower traits summarized in Table 1 (see below), the general shape of *L. incarnata s.l.* flowers was found to be different from those of the other analyzed taxa. In *L. algarviana*, *L. spartea* and the two subspecies of *L. viscosa*, a strongly folded lower lip was found to form a well-developed palate. By contrast, in *L. incarnata s.l.* the lower lip was found to be spread and scarcely folded. Therefore the palate was poorly developed in this group. On the other hand, *L. incarnata s.l.* corollas were found to be blue-violet with a small yellow spot in the lower lip (sometimes difficult to observe in herbarium specimens) and usually with darker violet veins. A similar pattern was found in *L. algarviana*, while corollas of *L. spartea* and the two subspecies of *L. viscosa* were completely bright yellow.

Seed characters: Seeds were reniform to trigonous, not laterally compressed, conspicuously cristate or ruminate-alveolate. Ridges were usually rounded (sometimes subacute, as in African populations of *L. incarnata s.l.* and *L. viscosa* subsp. *spicata*), predominantly oriented transverse to the main seed axis. Ridges were discrete (rarely anastomosed), in African and Huelva populations of *L. incarnata s.l.* and *L. viscosa* subsp. *viscosa*, whereas ridges were usually anastomosed in *L. spartea, L. viscosa* subsp. *spicata, L. algarviana* and *L. incarnata s.s.* Seed-coat cell sculpturing was formed by irregularly polygonal anticlinal walls and verruculate to rugulate periclinal walls. Periclinal wall of testa-cells formed a marginal papilla, usually rounded, towards the ridge-apex in all the studied species. We found median papillae (up to 11.5 μm long), which occupy a median position on the periclinal wall, only in some specimens of *L. algarviana* and in all the specimens of *L. incarnata s.l.* from Huelva (Fig. 2).

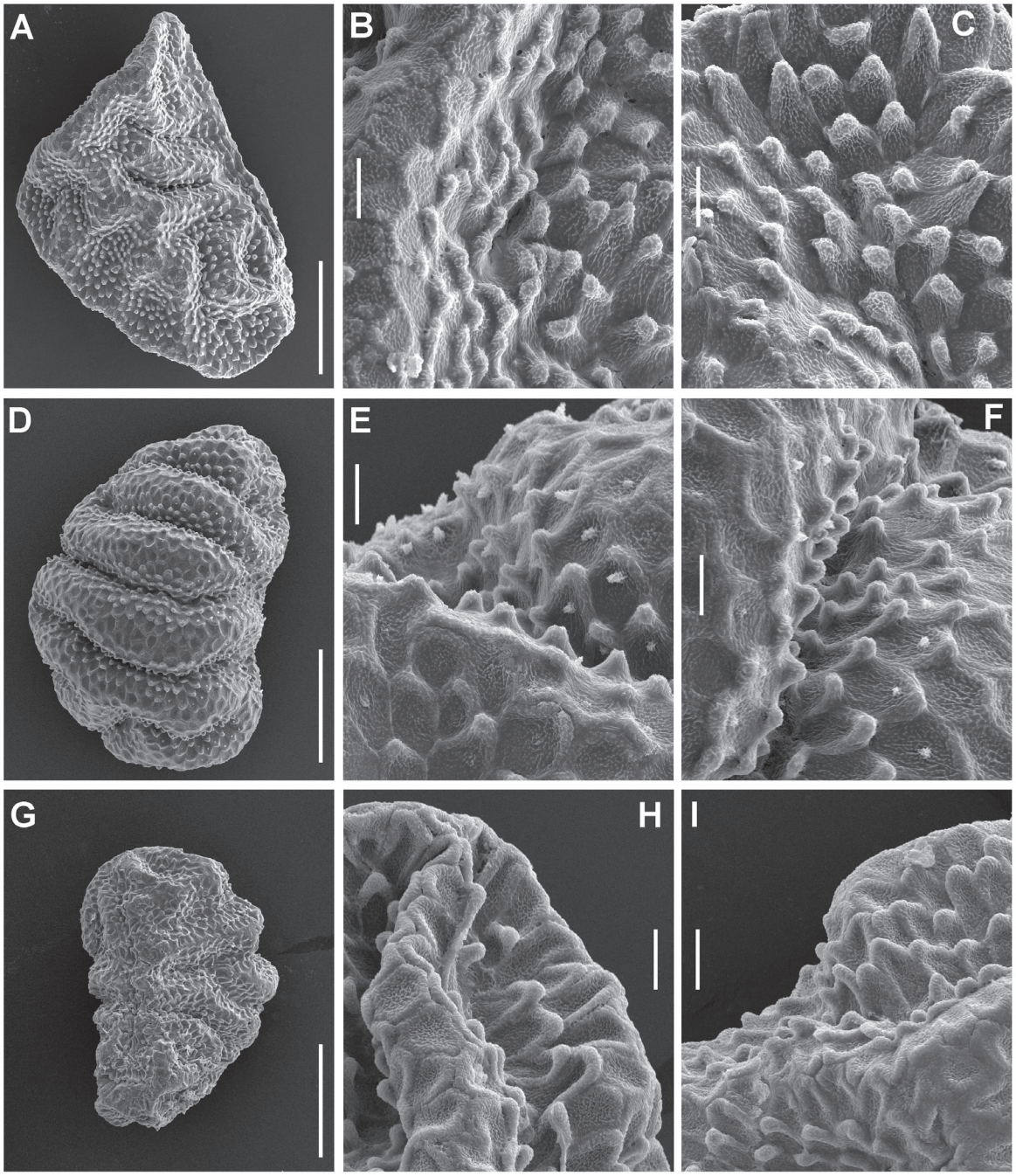


Fig. 2. Scanning electron micrographs of seeds of the *L. incarnata* complex: A-C, *L. incarnata s.s.* (Portugal, Beira Baixa, Serra da Malacata, MA 302210); D-F, *L. incarnata s.l.* from southwestern Spain (= *L. onubensis*) (Spain, Huelva, BCB); G-I, *L. incarnata s.l.* from Morocco (= *L. mamorensis*) (Morocco, Mamora, MA 109568) . Scale bars: A, D, G = 200 µm; B, C, E, F, H, I = 20 µm.

Statistical analysis of morphological characters

The PCoA of quantitative and qualitative traits for all taxa (Fig. 3A) retrieved four well-differentiated groups: three of them corresponded to specimens of *L. spartea*, *L. viscosa* subsp. *viscosa* and *L. viscosa* subsp. *spicata* respectively, while all specimens of *L. incarnata s.l.* were intermingled in the fourth group (Fig. 3A). The first two axes represented 17% of the variability. MANOVA of quantitative characters for all studied taxa revealed significant differences among them (Willk's Lambda = 0.003; F = 6.983; df = 95; P < 0.001). Although descriptive statistics and box plots did not show clear differentiation among taxa, as shown by the overlapping of all quantitative characters studied, the Tukey test revealed significant differences for various characters when comparing different pairs of taxa (results not shown).

In the PCoA analysis of *L. incarnata s.l.* populations (Fig. 3B), no clear groupings were obtained. However, samples from the three *a priori* population groups broadly occupied different places in the diagram, and there was no overlapping except for certain specimens. In this case the first two axes represented the 31% of the variability. The MANOVA performed for *L. incarnata s.l.* specimens alone showed significant differences with respect to morphology among the three population groups (Willk's Lambda = 0.010; F = 8.792; df = 40; P < 0.001). The Tukey test (Table 2) detected significant differences (P < 0.01) in trichome length between the three *L. incarnata s.l.* population groups, as also suggested by the box plots (Fig. 4). The test also revealed significant differences between *L. incarnata s.s.* and Moroccan/Huelva populations for five characters (leaf length on fertile shoots, fruit pedicel length, floral and fruit calyx-lobe length and bract length) (Table 2). Moroccan specimens displayed significant differences with those of *L. incarnata s.s.* for a total of seven traits, all of them vegetative except for the calyx-lobe length of flowers and fruits. Specimens from Huelva were significantly different from those of *L. incarnata s.s.* for 15 characters, and from Moroccan specimens for 11 characters. A total of 10 significantly different characters, including five floral traits, separated Huelva specimens from both Moroccan and *L. incarnata s.s.* specimens. All of them, except for trichome length, displayed non-significant differences between Moroccan and *L. incarnata s.s.* specimens.

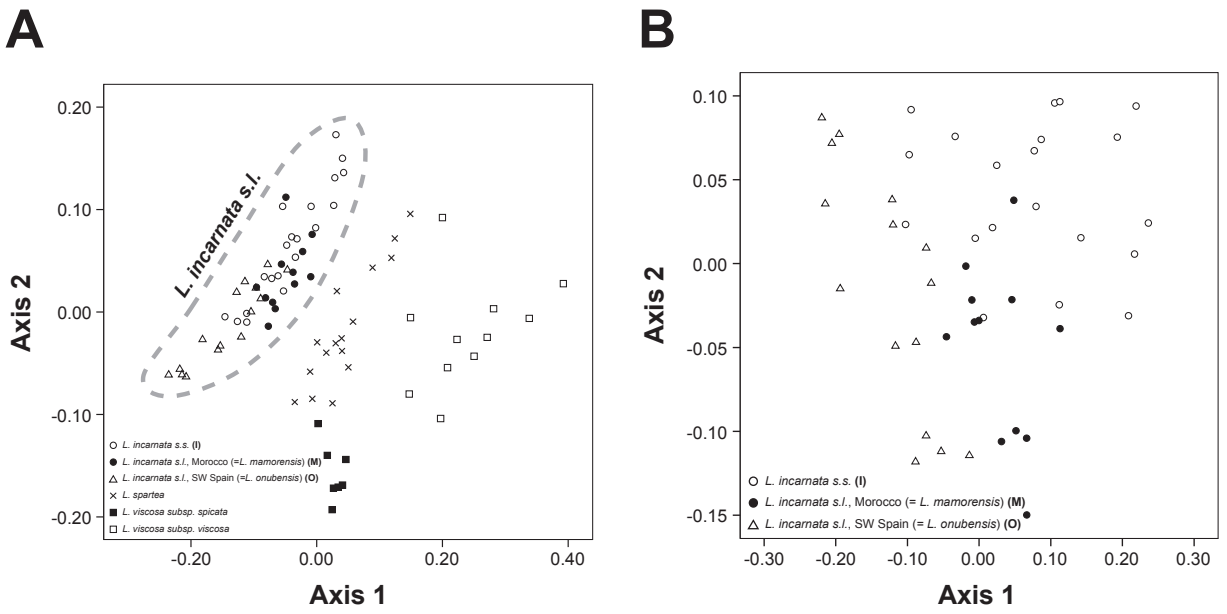


Fig. 3. Results of principal coordinate analysis (PCoA). A, biplot diagram resulting from the PCoA of specimens from all studied taxa of *Linaria* sect. *Versicolores*; B, biplot of the PCoA performed only with specimens of the *L. incarnata* complex.

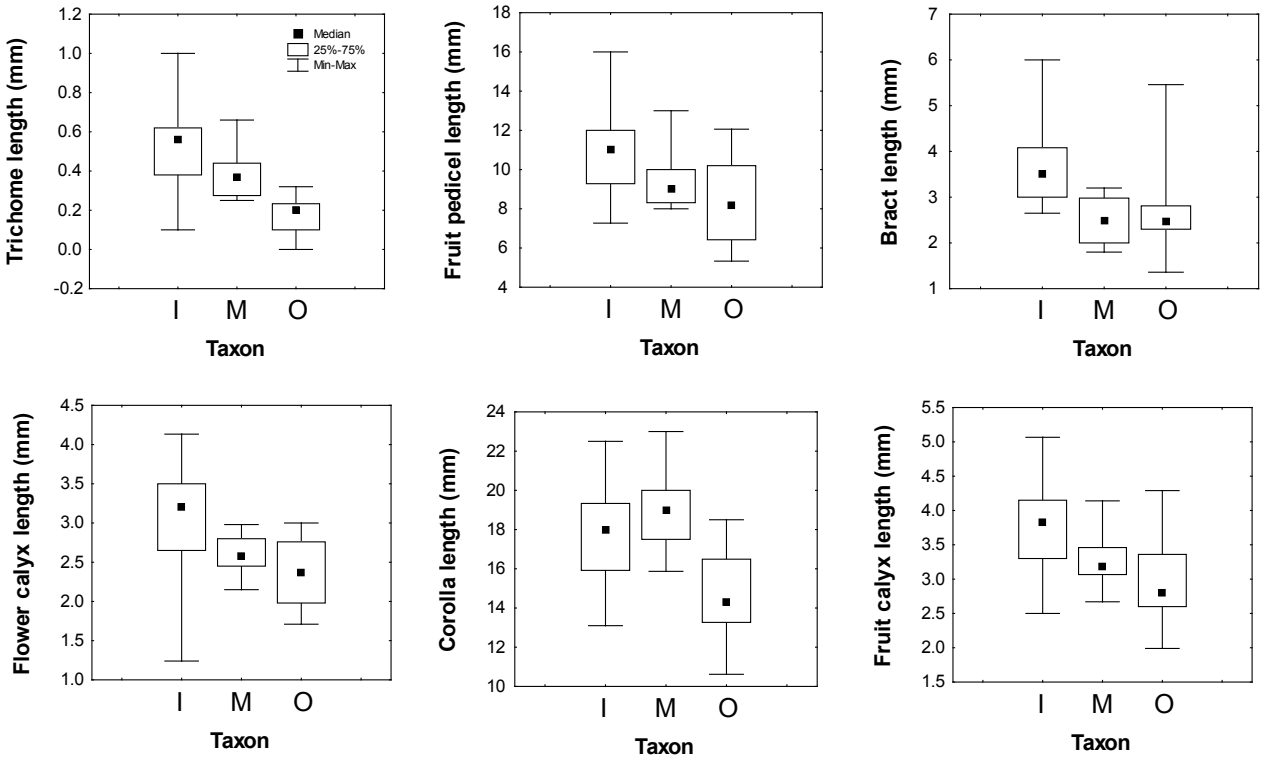


Fig. 4. Box plots for the most discriminant morphological characters between population groups of the *Linaria incarnata* complex. Population groups: I = *L. incarnata s.s.,* M = *L. incarnata s.l.* from Morocco (= *L. mamorensis*), O = *L. incarnata s.l.* from southwestern Spain (= *L. onubensis*).

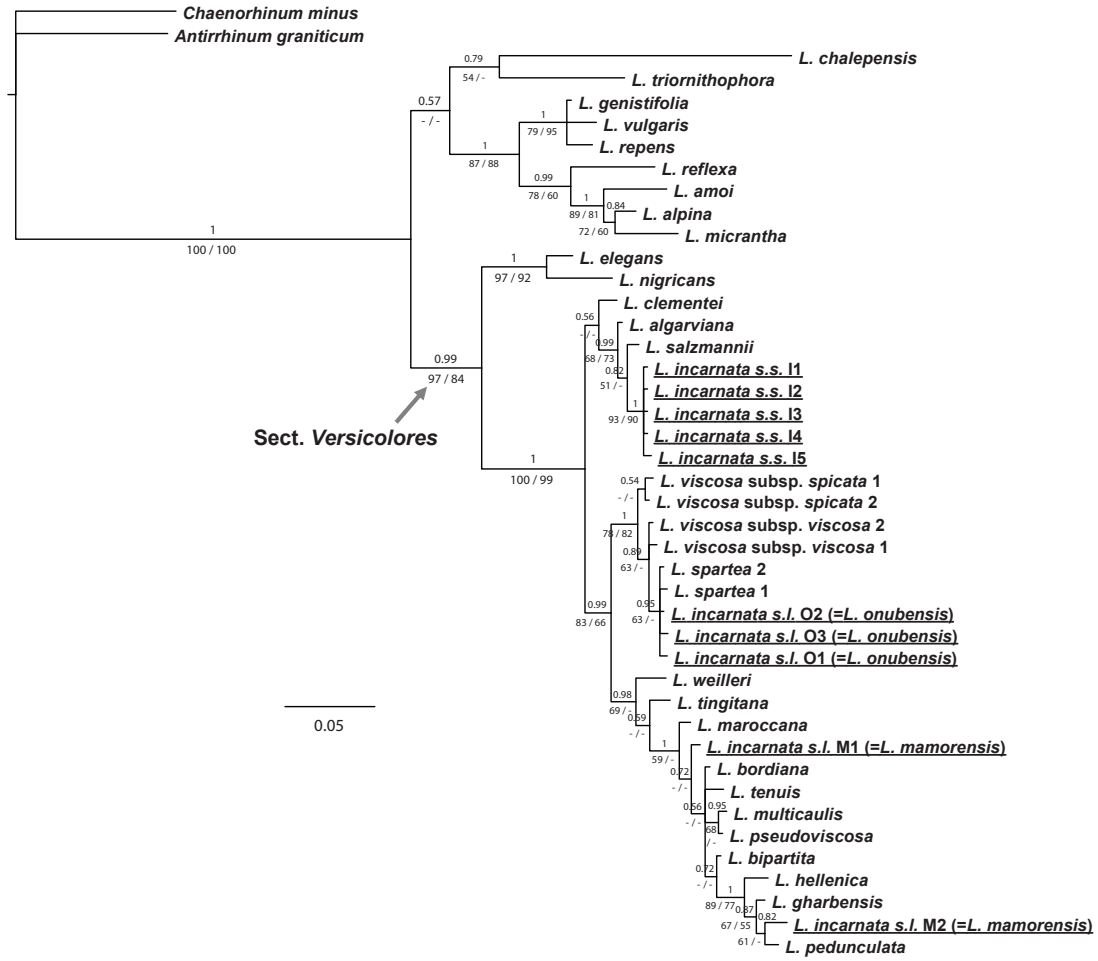
Phylogenetic analysis

The characteristics of the four sequenced cpDNA regions are summarized in Table 3. The aligned length was 603 bp for the ITS dataset and 1989 bp for the combined cpDNA dataset (730 bp for *rpl*32-*trn*L^{UAG}; 601 bp for *trn*S-*trn*G; and 658 bp for *ndh*F). The BI, ML and MP analyses yielded congruent topologies, except for some poorly supported clades. Lower resolution and support values were generally obtained in the MP analyses than in the ML and BI analyses. The 50% majority-rule consensus trees of the Bayesian analyses with ML and MP bootstrap supports are shown in Fig. 5.

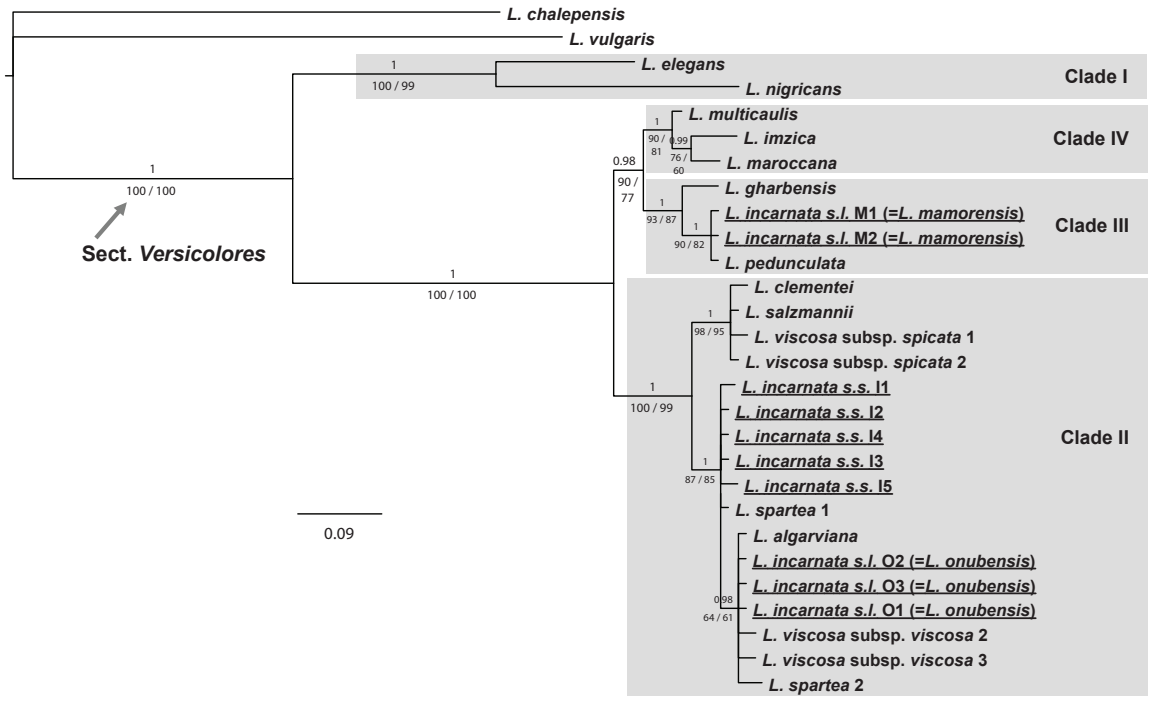
In the ITS analysis (Fig. 5A), *L. incarnata s.l.* was clearly retrieved as polyphyletic. Iberian specimens were nested within two distinct Iberian clades. The five Iberian individuals of *L. incarnata s.s.* (excluding Huelva populations) clustered together (PP = 1; ML-BS = 93%; MP-BS = 90%) within a clade also including *L. salzmannii* and *L. algarviana* (PP = 0.99; ML-BS = 68%; MP-BS = 73%), while the three individuals from Huelva grouped with individuals of *L. spartea* within a larger clade also including both sampled subspecies of *L. viscosa* (PP = 1; ML-BS = 78%; MP-BS = 82%). On the other hand, the two north African individuals were included within a mostly northern African clade (PP = 0.98; ML-BS = 69%; MP-BS < 50%). Relationships within this clade were poorly resolved.

The cpDNA phylogeny (Fig. 5B) yielded the same four major clades (I-IV) of *Linaria* sect. *Versicolores* reported by Fernández-Mazuecos & Vargas (2011). While largely incongruent with the ITS topology, the plastid tree also showed the *L. incarnata* complex as polyphyletic, and the same three groups of individuals were retrieved. The two northern African specimens grouped with other taxa from the same geographic region (*L. gharbensis*, *L. pedunculata*) within clade III (PP = 1; ML-BS = 93%; MP-BS = 87%). Within the Iberian clade, two well-supported sister clades were obtained: the first one included *L. clementei*, *L. salzmannii* and *L. viscosa* subsp. *spicata* (PP = 1; ML-BS = 98%; MP-BS = 95%), while the second one was formed by *L. spartea*, L. *viscosa* subsp. *viscosa*, L. *algarviana* and all Iberian samples of *L. incarnata s.l.* (PP = 1; ML-BS = 87%; MP-BS = 85%). Within the latter lineage, the three *L. onubensis* individuals were nested in a sub-lineage together with *L. algarviana*, *L. viscosa* subsp. *viscosa* and one individual of *L. spartea* (PP = 0.98; ML-BS = 64%; MP-BS = 61%), while the five individuals of *L. incarnata s.s.* and the remaining individual of *L. spartea* were found to be in a more basal position.

A. ITS


Chaenorhinum minus
Antirrhinum graniticum
L. chalepensis
L. triornithophora
L. genistifolia
L. vulgaris
L. repens
L. reflexa
L. amoi
L. alpina
L. micrantha
L. elegans
L. nigricans
L. clementei
L. algarviana
L. salzmannii
L. incarnata s.s. I1
L. incarnata s.s. I2
L. incarnata s.s. I3
L. incarnata s.s. I4
L. incarnata s.s. I5
L. viscosa subsp. spicata 1
L. viscosa subsp. spicata 2
L. viscosa subsp. viscosa 2
L. viscosa subsp. viscosa 1
L. spartea 2
L. spartea 1
L. incarnata s.l. O2 (=L. onubensis)
L. incarnata s.l. O3 (=L. onubensis)
L. incarnata s.l. O1 (=L. onubensis)
L. weilleri
L. tingitana
L. maroccana
L. incarnata s.l. M1 (=L. mamorensis)
L. bordiana
L. tenuis
L. multicaulis
L. pseudoviscosa
L. bipartita
L. hellenica
L. gharbensis
L. incarnata s.l. M2 (=L. mamorensis)
L. pedunculata
Sect. Versicolores
0.05

B. cpDNA


L. chalepensis
L. vulgaris
L. elegans
L. nigricans
Clade I
L. multicaulis
L. imzica
L. maroccana
Clade IV
L. gharbensis
L. incarnata s.l. M1 (=L. mamorensis)
L. incarnata s.l. M2 (=L. mamorensis)
L. pedunculata
Clade III
L. clementei
L. salzmannii
L. viscosa subsp. spicata 1
L. viscosa subsp. spicata 2
L. incarnata s.s. I1
L. incarnata s.s. I2
L. incarnata s.s. I4
L. incarnata s.s. I3
L. incarnata s.s. I5
L. spartea 1
L. algarviana
L. incarnata s.l. O2 (=L. onubensis)
L. incarnata s.l. O3 (=L. onubensis)
L. incarnata s.l. O1 (=L. onubensis)
L. viscosa subsp. viscosa 2
L. viscosa subsp. viscosa 3
L. spartea 2
Clade II
Sect. Versicolores
0.09

Discussion

Our morphological and phylogenetic results confirmed previous statements about the taxonomic complexity of the *L. incarnata* group (Pau, 1933; Sutton, 1988). All specimens of *L. incarnata s.l.* share a similar overall appearance with narrow-tubed, blue-violet to lilac corollas (Fig. 6), the same habit (annual) and the same type of leaves and fruits. In addition, all populations seem to be calcifuge and to usually occur in sandy grasslands. However, results from this study illustrate that specimens of *L. incarnata s.l.* from Huelva province (southwestern Spain) and Morocco are significantly distinct from those of the rest of Spain and Portugal for several vegetative, floral and fruit traits. In spite of the significant differences found for numerous quantitative traits (Table 2), the overlapping of measured values (Table 1; Fig. 4) makes them poorly discriminant from a taxonomic standpoint. In contrast, qualitative characters were found to be consistent as key traits to discriminate specimens of *L. incarnata s.l.* population groups. Indumentum density, trichome structure and size, seed shape and seed-coat sculpturing, together with the distinct geographic distributions (Fig. 7), and some ecological preferences (see below) justified the recognition of *L incarnata s.l.* populations from Huelva and Morocco as two different and independent species of genus *Linaria*. Accordingly, we assign populations in southwestern Spain to *Linaria onubensis* Pau –currently treated as a synonym of *L. incarnata* (Valdés, 1987)–,

Fig. 5. Phylogenetic analyses of the *L. incarnata* complex based on nuclear and plastid DNA sequences. A, phylogenetic relationships among *Linaria* sect. *Versicolores* species based on nuclear ITS sequences, including ten samples of *L. incarnata s.l.* The fifty percent majority-rule consensus tree obtained in the Bayesian analysis is shown. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood/maximum parsimony bootstrap supports. A hyphen (-) indicates no bootstrap support over 50%. Populations of the same species are named as in Table S1. B, phylogenetic relationships among *Linaria* sect. *Versicolores* species based on concatenated cpDNA sequences (*rpl*32-*trn*L^{UAG}, *trn*S-*trn*G, *ndh*F), with special emphasis on Iberian taxa (clade II). Ten samples of *L. incarnata s.l.* are included. The fifty percent majority-rule consensus tree obtained in the Bayesian analysis is shown. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood/maximum parsimony bootstrap supports. Populations of the same species are named as in Table S1. Clades are named as in Fernández-Mazuecos & Vargas (2011).

and propose a new name (*Linaria mamorensis*) for Moroccan populations of *L. incarnata s.l.* (Figs. 6, 7; see below).

Seed shape and microsculpturing of seed coat have been shown to be informative characters in the taxonomy of the tribe Antirrhineae (Elisens & Tomb, 1983; Elisens, 1985; Sutton, 1988). This is also true for *Linaria*, as shown by Sutton (1988), Viano (1978b, c) and several studies on different sections and groups of the Iberian Peninsula (Juan *et al.*, 1999; Segarra & Mateu, 2001a, b; Sáez *et al.*, 2008). Seed traits also vary among the newly recognized *Linaria* species (Fig. 2). In the *L. incarnata* complex, only *L. onubensis* has a median papilla in the periclinal wall of testa cells (Fig. 2E, F; see also Juan *et al.*, 1999). According to Sutton (1988), this median papilla can occasionally appear in *L. spartea* and *L. algarviana*, and it is usually present in *L. viscosa*. However, the median papilla is completely absent in *L. incarnata s.s.* (Fig. 2B, C; see also Sutton, 1988) and *L. mamorensis* (Fig. 2H, I; see also Viano, 1979). Thereby, the presence of a median papilla on ridge testa cells of *L. onubensis* is a relevant character for its differentiation from *L. incarnata s.s.* and *L. mamorensis*, together with its reniform seed shape *versus* the subtrigonous shape of *L. incarnata s.s.* In the case of *L. mamorensis*, seeds differ from those of *L. incarnata s.s.* mainly because of its reniform shape transversely ridged (subtrigonous and ruminate-alveolate in *L. incarnata s.s.*).

Although our findings regarding seed shape and seed-coat microsculpturing are consistent with previous SEM observations of *L. incarnata s.l.* seeds from diverse locations (Viano, 1979; Sutton, 1988; Juan *et al.*, 1999), it has to be acknowledged that these traits may be variable within taxa. This has been reported for several species complexes of section *Supinae*, including those of *L. verticillata*, *L. depauperata* and *L. supina* (Segarra & Mateu, 2001b; Sáez *et al.*, 2004; Sáez & Crespo, 2005). Fortunately, indumentum characters (Segarra & Mateu, 2001b; Sáez & Crespo, 2005) may be additionally used to discriminate between the newly-recognized species, as shown by statistical analyses (Table 1; Fig. 4). Inflorescences of *L. incarnata s.s.* have a denser indumentum and longer trichomes than those of *L. mamorensis* and *L. onubensis* (Tables 1, 2; Fig. 4) and trichome structure of *L. onubensis* differs from that of *L. incarnata s.s.* and *L. mamorensis* (Fig. 1). Indumentum density and trichome length were the most reliable traits to discriminate between *L. incarnata, L. mamorensis* and *L. onubensis* (Table 4).

Fig. 6. Representative specimens of the three recognized species: A, living inflorescence of *L. incarnata s.s.* (Spain, Badajoz, Alburquerque); B, living inflorescence of *L. onubensis* (Spain, Huelva, Valverde del Camino); C, holotype specimens of *L. mamorensis* (Morocco, road from Kenitra to Khemisset).

Table 4. Main morphological differences between *Linaria incarnata*, *L. mamorensis* and *L. onubensis*. Measurements are in milimeters.

	L. incarnata	*L. mamorensis*	*L. onubensis*
Inflorescence	densely glandular-pubescent	sparsely glandular-pubescent	sparsely glandular-pubescent
Hairs			
length	(0.1) 0.3-1	0.1-0.6	0.1-0.3
transverse walls	purple to violet	hyaline, rarely rose	hyaline
Corolla			
length	13.1-22.5	15.8-23	10.6-18.5
adaxial lip sinus	(3.5) 4.3-9	5-7.3	(0.9) 2-4.8
abaxial lip sinus	(1.5) 2.8-5 (6)	1.7-4 (5)	0.8-2.5
Seed			
shape	subtrigonous or trigonous-reniform, ruminate-alveolate	reniform, transversely ridged	reniform, transversely ridged
median papilla	absent	absent	present

Our taxonomical proposal for upgrading *L. onubensis* to species level and recognizing *L. mamorensis* as a new species was also supported by phylogenetic results. Despite topological incongruence between phylogenetic trees based on plastid and nuclear DNA sequences (Fig. 5), polyphyly of the *L. incarnata* complex was unambiguously recovered from all analyses. Incongruence can be ascribed to multiple causes not analyzed here, including incomplete lineage sorting and hybridization, which are particularly prevalent in recently-diversified lineages, including *Linaria* (Blanco-Pastor *et al.*, 2012). Nevertheless, individuals of the three species recognized here were consistently retrieved at distinct phylogenetic positions in both analyses. This fact, together with the morphological evidence presented above, suggests that the three clusters of individuals constitute distinct evolutionary lineages.

Interestingly, non-monophyly of *L. mamorensis* was obtained in the ITS tree. We do not consider that this is enough evidence for recognition of multiple entities in northern Africa. First, northern African populations studied here have been found to be morphologically homogeneous, which does not suggest the presence of multiple taxa in this region (as opposed to the Iberian Peninsula). And second, non-monophyly of intra-specific sequences at shallow phylogenetic levels is not surprising given the recent diversification of *Linaria* sect. *Versicolores* (Fernández-Mazuecos & Vargas, 2011; Chapter 3; Chapter 4). According to coalescence theory, evolution of species monophyly is four times slower in nuclear than in uniparentally inherited plastid sequences, due to differences in effective population size (Palumbi *et al.*, 2001). Therefore, time

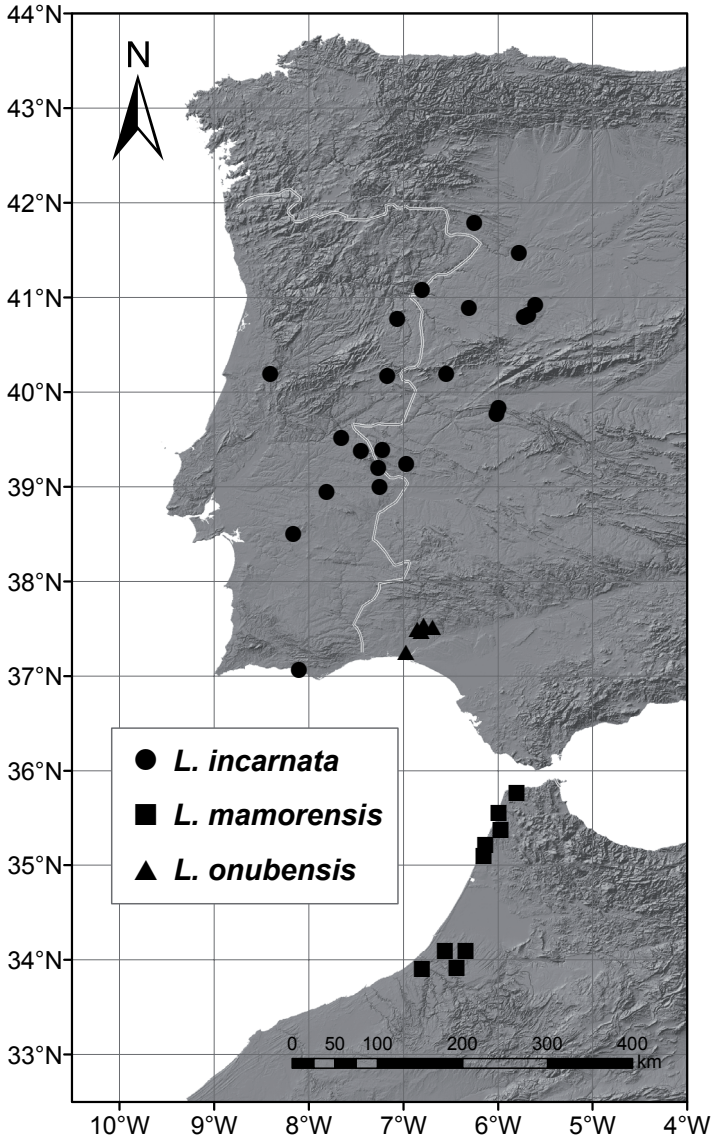


Fig. 7. Geographic distribution of representative specimens belonging to the three recognized species.

since speciation may not have been enough for the coalescence of intra-specific ITS sequence copies of *L. mamorensis*. Alternatively, introgressive hybridization between *L. mamorensis* and closely-related species of subsect. *Versicolores* may have occurred (Viano, 1978a; Sutton, 1988; Sáez & Bernal, 2009), which could have also led to polyphyly of *L. mamorensis* in the ITS tree (Álvarez & Wendel, 2003; Nieto-Feliner & Rosselló, 2007).

The polyphyly of the *L. incarnata* complex herein reported suggests convergent evolution (*sensu* Scotland, 2011) of *L. incarnata*-type morphology in the course of the evolution of *Linaria*

sect. *Versicolores*. *L. incarnata s.s.*, *L. mamorensis* and *L. onubensis*, as defined here, could be considered to constitute a complex of "cryptic species" *sensu* Bickford *et al.* (2007), i.e. distinct species that have been erroneously classified under one species name due to poor morphological differentiation. In addition to revealing the three taxa as distinct evolutionary lineages, we have reported significant morphological differences, previously overlooked (Viano, 1969; Valdés, 1987) or not analyzed in detail (Sutton, 1988). This confirms that combination of different sources of evidence (including biometric and phylogenetic data) is needed for a well-supported species delimitation (De Queiroz, 2007; Wiens, 2007).

Taxonomic treatment

Linaria incarnata (Vent.) Spreng., Syst. Veg. ed. 16, 2: 796 (1825)

≡ *Antirrhinum incarnatum* Vent. in Lam., Encycl. 4: 364 (1797)

Ind. loc.: "Cette jolie plante croît en Espagne, d'où elle fut raportée par m. Antoine de Jussieu"

Lectotype (Laínz, 1966: 56): Portugal, circa Pombal, IV-1717, s.r. (B)

= *L. linogrisea* Hoffmanns. & Link, Fl. Port. 1: 240. t. 11 (1809)

≡ *Antirrhinum linogriseum* Brot., Phytogr. Ins. Sel. 2 (1816)

≡ *L. bipartita* subsp. *linogrisea* (Hoffmanns. & Link) Maire in Jahand. & Maire, Cat. Pl. Maroc 3: 673 (1934)

Ind. loc.: "Fréquente dans les contrées sablonneuses de l'Algarve"

Type material: Not seen.

Description: Annual, glabrous below, densely glandular-pubescent in the inflorescence (hairs (0.1)0.3-1 mm long, with purple to violet transverse walls 3.2-7.1 μm width). Fertile stems 15-55 cm long, usually erect, simple or somewhat branched. Sterile stems 1-6 cm, procumbent to ascendent. Leaves of fertile stems 5.8-31.4 x 0.48-1.5 mm, linear or linear-lanceolate, obtuse, flat or involute, alternate; leaves of sterile stems 3-8.3 x 0.5-2.1 mm, linear-lanceolate to elliptic, obtuse, opposite or verticillate. Inflorescence 3.5-29 cm long, with 3-20 flowers, usually lax (sometimes somewhat dense) in flower, lax in fruit. Bracts 2.7-6 x 0.6-1.5 mm, ovate to lanceolate, acute or subacute. Pedicels 4-7.5 mm in flower, 6.5-16 mm in fruit, erecto-patent

to erect, not adnate to inflorescence axis. Calyx lobes subequal, 3-4.5 x 0.5-1 mm in flower, 3.8-5 x 0.7-1.2 mm in fruit, lanceolate to linear-lanceolate, acute or subacute. Corolla 13.1-22.5 mm, lilac, violet, or blue-violet, adaxial lip sinus (3.5)4.3-9 mm, abaxial lip sinus (1.5)2.8-5(6) mm; spur 7-13.4 mm, straight or curved, more or less equaling or longer than the rest of the corolla. Style deeply bifid (rarely trifid). Capsule 2-4.7 x 2-4 mm, oblong, usually glabrous; loculi subequal, each loculus dehiscing by 3 teeth. Seeds 0.5-0.7 x 0.4-0.5 mm, subtrigonous, ruminate-alveolate, greyish to blackish-grey; ridges low, rounded, anastomosed; periclinal wall of testa-cells verruculate, the margin raised, produced into rounded marginal papilla towards ridge apex; median papilla absent.

Distribution: Endemic to Western Iberian Peninsula (Portugal and Spain).

Habitat: Grassland and open shrubby formations and path margins, usually on siliceous sandy soils. Altitudinal range: 200-850 m.

Phenology: March-May.

Illustration: Viano (1978b: 83); Hoffmannsegg & Link (1813: pl. 41) [sub *L. linogrisea*].

Observations: *Linaria incarnata* bears a general resemblance with *L. elegans*. The ranges of both species overlap in western Iberian Peninsula, and they are sometimes confused in herbaria. Both species can be easily distinguished by the conspicuously bifid (or sometimes trifid) style with discrete stigmatic areas of *L. incarnata*, as opposed to the simple style and emarginated stigma of *L. elegans*. In addition, the corolla of *L. incarnata* usually displays a yellow spot in the palate, which is always missing in *L. elegans*. Even though *L. incarnata* is broadly sympatric with the yellow-flowered *L. spartea*, we have not observed putative hybrids between them, neither in herbaria nor in the field. On the other hand, hybrids with *L. algarviana* have been reported in southern Portugal (Viano, 1978b).

2. *Linaria mamorensis* Mazuecos, Vigalondo & L.Sáez **sp. nov.**

Holotype: Marruecos, ctra. Kenitra-Khemisset, a unos 20 km de Kenitra, charcas temporales en suelos arcilloso-arenosos, llanura abierta, 17/IV/2006, S. Martín-Bravo (34SMB06), I. Pulgar, F.J. Fernández, G.C. Mazo (MA 856684)

Diagnosis: *L. mamorensis* is similar to *L. incarnata*, but differs by the following features: the inflorescence is sparsely glandular-pubescent (hairs 0.1-0.6 mm long) (*vs.* densely glandular-pubescent in the inflorescence, with hairs (0.1)0.3-1 mm long); seeds reniform, transversely ridged (*vs.* subtrigonous, ruminate-alveolate), with ridges discrete, rarely anastomosed (*vs.* anastomosed).

Description: Annual, glabrous below, sparsely glandular-pubescent in the inflorescence (hairs 0.1-0.6 mm long, with hyaline –rarely rose– transverse walls 1.7-5.2 μm width). Fertile stems 14-62 cm long, usually erect, simple or somewhat branched. Sterile stems 1-9 cm, procumbent to ascendent. Leaves of fertile stems 8-32(45) x 0.5-1.6(2) mm, linear or linear-lanceolate, obtuse, flat or involute, alternate; leaves of sterile stems 2.7-11 x 0.8-1.7 mm, linear-lanceolate to narrowly elliptic, obtuse, verticillate. Inflorescence 2-25 cm long, with 3-23 flowers, lax (sometimes somewhat dense) in flower, lax in fruit. Bracts 1.8-3.2 x 0.4-1 mm, linear to linear-lanceolate, acute or subacute. Pedicels 5-13 mm in flower, 8-14 mm in fruit, erecto-patent to erect, not adnate to inflorescence axis. Calyx lobes subequal, 2.1-3.5 x 0.5-0.9 mm in flower, 2.6-4.5 x 0.5-1.1 mm in fruit, lanceolate to linear-lanceolate, acute or subacute. Corolla 15.8-23 mm, lilac, violet, or blue-violet, adaxial lip sinus 5-7.3 mm, abaxial lip sinus 1.7-4(5) mm; spur 8.8-13.1 mm, straight or curved, more or less equaling or longer than the rest of the corolla. Style deeply bifid. Capsule 3.2-4.2 x 2.7-3.5 mm, oblong, glabrous or sparsely hairy (hairs 0.1-0.3 mm long); loculi subequal, each loculus dehiscing by 3 teeth. Seeds 0.4-0.6 x 0.3-0.4 mm, reniform, transversely ridged, grayish to blackish-grey; transverse ridges 6-7(8), rounded to subacute, discrete or rarely anastomosed; periclinal wall of testa-cells verruculate, the margin raised, produced into rounded marginal papilla towards ridge apex; median papilla absent.

Distribution: Endemic to NW Morocco.

Habitat: Grassland and open woodland, mainly on siliceous sandy soils. Altitudinal range: 5-200 m. The type locality is a *Quercus suber* woodland with therophytic communities intermingled with small temporal ponds.

Phenology: March-May.

Observations: As circumscribed here, *L. mamorensis* includes populations from lowland areas of northwestern Morocco. Populations further south in higher lands of the High Atlas (Marrakech area) and the Anti Atlas were also included in *L. incarnata* by previous authors (Viano, 1969; Sutton, 1988), and have sometimes received taxonomic recognition at various taxonomic ranks (Viano, 1969; Sutton, 1988). However, we are not including them in *L. mamorensis* because morphological traits and phylogenetic relationships (Fernández-Mazuecos & Vargas, 2011; Chapter 3) indicate affinity with *L. bipartita* and *L. maroccana*. *Linaria viviesiae* is a very rare taxon from the Oulmes region (Fennane & Ibn Tattou, 1998), clearly distinguished from *L. mamorensis* by its minute corolla (10 mm long), similar to those of *L. arvensis* and *L. micrantha* (Emberger, 1935; Gómiz, 2004).

3. *Linaria onubensis* Pau in Broteria, Ser. Trimestr. 2: 50 (1933)
Ind. Loc.: "Valverde del Camino (Huelva) (E. Gros)"
Type material: Valverde del Camino, 13.V.1931, E. Gros det. Dr. C. Pau (MA 109497)

Description: Annual, glabrous below, sparsely glandular-pubescent in the inflorescence (hairs 0.1-0.3 mm long, with hyaline transverse walls 0.9-2.8 μm width). Fertile stems 19-64 cm long, erect to suberect. Sterile stems 2-14.5 cm, procumbent to ascendent. Leaves of fertile stems 5.8-31.4 x 0.2-1.2 mm, linear, obtuse, flat or involute, alternate; leaves of sterile stems 2-9 x 0.7-1.9 mm, linear-lanceolate to narrowly elliptic, obtuse, verticillate. Inflorescence 3.4-23.4 cm long, with 3-21 flowers, lax in flower and fruit. Bracts 1.3-5.5 x 0.5-1 mm, lanceolate, acute or obtuse. Pedicels 2.8-9 mm in flower, 5.3-12.1 mm in fruit, erecto-patent to erect, not adnate to inflorescence axis. Calyx lobes subequal, 1.7-3 x 0.4-1.1 mm in flower, 1.9-4.3 x 0.5-1 mm in fruit, linear-lanceolate, subacute or obtuse. Corolla 10.6-18.5 mm, blue-violet, sometimes pale lilac, adaxial lip sinus (0.9)2-4.8 mm, abaxial lip sinus 0.8-2.5 mm; spur 5.9 - 9.8 mm, straight or curved, more or less equaling the rest of the corolla. Style deeply bifid. Capsule 2.1-3.6 x 2-3.5 mm, oblong, glabrous; loculi subequal, each loculus dehiscing by 3 teeth. Seeds 0.4-0.6 x 0.3-0.46 mm, reniform, transversely ridged, grayish to blackish-grey; transverse ridges 5-6(7), rounded, discrete or rarely anastomosed; periclinal wall of testa-cells verruculate, the margin

raised, produced into rounded marginal papilla towards ridge apex; median papilla 2.5-11.5 μm long.

Distribution: Endemic to southwestern Spain (Huelva province).

Habitat: Grasslands under dispersed trees (*Quercus suber*, *Q. ilex s*ubsp. *ballota* and *Pinus pinea*) and open shrubby formations, on siliceous sandy soil. Altitudinal range: 3-280 m.

Phenology: March-May.

Illustration: Valdés (1987: 512) [sub *L. incarnata*].

Observations: This species usually occurs in artificially open areas, which means that the abandonment of crops, the disappearance of grasslands and the consequent increase of shrubs and bushes could negatively affect its establishment. Furthermore, in all studied localities, *L. onubensis* is only found in alluvial sandy soils mixed with pebbles of acid nature. According to geological maps (Contreras & Santos, 1972; De Torres, 1972; Ramírez & Navarro, 1972; Fernández *et al.*, 1983; Ramírez & Leyva, 1983), such substrate corresponds to Quaternary soils of conglomerates and red sands, which is a restricted type of substrate in the study area, and also to a more common one of Miocene conglomerates and sands. This implies that *L. onubensis* could be an edaphism. Consistent with this and given that this species is endemic to an apparently reduced area (present in 6 UTM 1x1 km^2 squares), more field research is needed to evaluate the conservation status of *L. onubensis* populations and their possible threats. A small population detected in a gardening area of Marismas del Odiel seems to have an artificial origin. Hybrid individuals with *L. spartea* are also present in the same population.

Key to species

This is an amendment for the key in Sutton (1988) to accommodate *Linaria mamorensis* and *L. onubensis.*

119. Leaves of sterile stems linear to narrowly elliptic, obtuse; racemes usually lax in flower, lax in fruit **120**

119. Leaves of sterile stems elliptic, subacute to obtuse; racemes dense in flower, scarcely elongating in fruit ..... ***L. bordiana***

120. Inflorescence densely glandular-pubescent, covered by trichomes (0.1)0.3-1 mm long with purple to violet transverse walls; seed subtrigonous ..... ***L. incarnata***

120. Inflorescence sparsely hairy, trichomes 0.1-0.6 mm long with hyaline, rarely rose, transverse walls; seed reniform **121**

121. Corolla 10.6-18.5 mm long; adaxial lip sinus (0.9)2-4.8 mm ..... ***L. onubensis***

121. Corolla 15.8-23 mm long; adaxial lip sinus 5-7.3 mm ..... ***L. mamorensis***

Acknowledgements

The authors thank Santiago Martín-Bravo for providing the type material of *L. mamorensis* and for information about the type locality; Juan Antonio Calleja and Enrique Sánchez-Gullón for field assistance and plant material of *L. onubensis*; Emilio Cano for laboratory assistance; and Charo Noya for assistance in the MA herbarium. This research was supported by the Spanish Ministry of Science and Innovation through project CGL2009-10031, and by the Spanish Ministry of Education through a FPU fellowship (AP2007-01841) to MFM.

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Supporting Information

Appendix S1. Specimens examined.

Linaria incarnata (Vent.) Spreng.

Portugal: Casa-Branca, IV-1915, G. Sampaio (MA 109574); Alto Alentejo, Serra del Mamede, próximo da torre Caldina, poussio sob. *Quercus suber*, V-1957, Malato-Beliz *et al.* (MA 179810, sub *L. bipartita*); Nisa, Nª Sra. de Graça, 4-V-1971, Malato-Beliz & Guerra (MA 302209); Nisa, Nª Sra. de Graça, 4-V-1971, Malato-Beliz & Guerra (SEV 10305); Beira Baixa, Penamacor, Serra da Malacata, 12-V-1970, J. Malato-Beliz & J.A. Guerra 8197 (MA 302210); Beira Interior, Pinhel, entre Ervas Tenras e Malta ao km 109,7, em terrenos incultos, matos e granitos, 28-VI-1970, Rogeira, Serra & Bernardino (MA 499656); Beira Litoral, Coimbra, pinhal de Marrocos, 26-V-1954, Matos & Marques (MA 302213); Coimbra, Pinhal de Marrocos, 26-III-1954, Matos & Marques (SEV 3965); Pinhais de Quarteira, 23-IV-1968, Bellot & Casaseca (MA 187813, sub *L. bipartita*).

Spain: Badajoz, ctra. de La Codosera, 29SPD54, 7-III-1977, P. Gómez Hdez. (MA 453484); Alburquerque, suelos arenosos de granitos,19-IV-1978, J.L. Pérez Chiscano (MA 330389); Cáceres, Torrejón el Rubio, encinares aclarados sobre suelos silíceos, 24-III-1982, Ladero, F. Navarro & C. Valle (MA 330441); borde carretera Torrejón el Rubio, 9-VI-1993, Pérez Chiscano (MA 531061); Carretera de Torrejón el Rubio a Trujillo, 12-IV-1977, Pérez Chiscano (MA 205819); Pantano del Tiétar, arenoso granítico, 280 m s.m., 21-IV-1987, A. Segura Zubizarreta nº 34555 (MA 580953); Pantano del Tiétar, arenoso-granítico, 280 m s.m., 21-IV-1987, A. Segura Zubizarreta nº34555 (MA 580661); Monfragüe, pantano del Tietar, arenoso-granítico, 280 m s.m., 21-IV-1987, A. Segura Zubizarreta (MA 581980); km 43 ctra. Torrejón el Rubio a Trujillo, suelos arenoso-lignoso pliocuaternario, 12-IV-1977, J.L. Pérez Chiscano (MA 330445); término de Santibáñez el Alto, suelo sobre granitos, 16-IV-1991, J.L. Pérez Chiscano (MA 493328); Salamanca, Aldea del Obispo, 22-IV-1976, E. Rico (MA 205820); Brincones, 6-V-1976, J. Sánchez (MA 330444); Berrocal de Huebra, 22-5-1976, Fernández Díez (MA 201537); Zamayón, 3-V-1977, J. Sánchez (MA 330446); entre Villares de Yeltes y Boada, 14-V-1978, F. Amich (MA 333261); El Cubo de Don Sancho, 28-V-1980, J. Fdez. Díez y F. Amich (MA 330443); Mozárbez, route vers Monterrubio de la Sierra, pelouses thérophytiques subnitrophiles (*Brometalia rubenti-tectorum* Rivas Goday & Rivas-Mart. 1963 em. nom.), UTM 30T TL 72, 820 m s.m., 15-V-1983, M. Ladero & F. J. González (MA 367688); encinares adehesados, El Cubo de Don Sancho, 28-V-1980, Fernández Díez & Amich (SEV 53108); Morille, suelos arenosos, TL 7421, 16-V-1998, de Paz (ABH 44296); Zamora, Las Chanas, in arvis incultis, 13-VI-1971, B. Casaseca (MA 423792, sub *L. bipartita*); Mayalde, 15-V-1983, X. Giráldez (MA 416266).

Linaria mamorensis Mazuecos, Vigalondo & L.Sáez

Morocco: Entre Rabat y Tiflet, forêt de la Mamora, alcornocales, 150 m, 15-V-1969, E. Paunero, E.F. Galiano, P. Gibbs & B. Valdés (SEV 94721); Forest between Tangier & Asilah, near sea level, *Quercus suber* forest on sandy soil, 6-IV-

1971, Davis 51300 (RNG 95103-447); Hab. In arenosis c. El Araix, l. El Mensali, ad 40 m, 8-II-1930, Font Quer Iter maroccanum 589 (MA 109567); Kenitra, entre Sidi-Yaha-du-Rharb y Sidi-Slimane, 9-IV-1983, J.A. Devesa, E.F. Galiano & S. Talavera (SEV 160821); Kenitra, sables, Mamora, littoral, IV-1933, J. Gattefossé (MA 109568); Larache, primavera de 1914, Pérez-Chiscano 41 (MA 109564); Mamora, Ain Jorra, sables, 1-V-1924, E. Jahandiez (MA 109565); Mamora, Kenita, sables, litoral, abril de 1933 (MA 109568, sub *L. bipartita*); Playa de Tánger, IV-1921, Pau (MA 109566); près aeroport Rabat-Salé, près de la forêt de la Mamora, 93 m, champs cultivés, sol sableux siliceux, 15-III-1977, M. Atbib (MA 3676867); près de la forêt de la Mamora, près Aéroport Rabat-Salé, sol sableux, siliceux, champs cultivé, 93 m s.m., 15-III-1977, M. Atbib (MA 367687); près de la forêt de la Marmora, près Aéroport Rabat-Salé (champs cultivé), sol sableux, siliceux, 15-III-1977, M. Atbib (RNG 95103-455); Rabat, 10 km W de Tiflete, sables d'une vigne, 1955, Ch. Sauvage 13828 (MA 303515, sub *L. bipartita*); reg. Rabat, 10 km W de Tiflete, sables d'une vigne, 22-VIII-1955, Sauvage 13828 (MA 303515); Salé, prov. de Salé, 7 km a l'E de Salé, route vers Meknès, Layayda. 34º01'22''N 6º44'49''W, alt. env. 50 m, pelouse rase pâturée, sur sable, piquetée de chardons, 19-III-1995, Lambidon & G. Van Den Sande (RNG s.n.); Tetuán, entre Asilah y Larache, Tnine-Sidi-El Yaman, eucaliptal sobre arenas, 20-IV-1988, Silvestre, G. Rowe & Vilches (RNG 95119-21); Tetuán, entre Asilah y Larache, Tnine-Sidi-El Yaman, eucaliptal sobre arenas, 20-IV-1988, Silvestre, G. Rowe & Vilches (SEV 160669).

Linaria onubensis Pau

Spain: Huelva, Valverde del Camino, 13-V-1931, Gros (MA 109495, MA 109496, MA 109497); entre Trigueros y Valverde, limos arenosos diluviales, 11-IV-1960, Rivas Goday *et al.* (MA 174583); Valverde del Camino, sobre suelos arenosos ácidos, 20-III-1978, Valdés (SEV 164017); Entre La Palma del Condado y Valverde del Camino, 20-VI-1978, Talavera & Valdés (MA 467392); Casa Batanero, 21-IV-2011, Vigalondo & Calleja (s.n.); El Saltillo, 21-IV-2011, Vigalondo & Calleja (s.n.); Fuente de la Corcha, 21-IV-2011, Vigalondo & Calleja (s.n.); La Dehesa, entre Beas y el desvío a Fuente de la Corcha, 22-IV-2011, Vigalondo & Calleja (s.n.); Marismas del Odiel, Centro de Visitantes, 22-IV-2011, Vigalondo & Calleja (s.n.); Riotinto-Campofrío, 17-IV-1980, Rivera *et al.* (MA 330468); Valverde del Camino, parque periurbano El Saltillo, 300 m, Vargas & Valcárcel (MA s.n.); Valverde del Camino, parque periurbano El Saltillo, 300 m, Fernández-Mazuecos (MA s.n.).

Linaria algarviana Chav.

Portugal: Algarve, Cabo de São Vicente, a W estrada, em solo arenoso, vermelho. 60 msm, 14-II-1941, Pinto da Silva (MA 110353); Cabo de São Vicente, a W estrada, em solo arenoso, vermelho. 60 msm, 14-II-1942, Pinto da Silva (MA 110354); Cabo de São Vicente, a W estrada, em solo arenoso, vermelho. 60 msm, 14-II-1943, Pinto da Silva (MA 110355); Cabo de São Vicente, 24-III-2009, M. Fernández-Mazuecos 11MF09 (MA).

Linaria spartea (L.) Chaz.

Portugal: Alto Alentejo, Elvas, Falcato, 13-IV-1978, Beliz & Guerra (MA 304340); Elvas, Verdu, Quinta de Sta. Rita, 31-III-1954, Guerra (MA 304315); Mora, a 7 km de Pavia, 18-IV-1987, Moura (MA 395573); Baixo Alentejo, Serjoa-Pias, Lagares de Burrico, 12-IV-1949, Fontes & Rainha (MA 304319); Estremadura, Sesimbra, Alfarim, próximo al Lago de Albufeira, 2-VI-1971, Beliz & Guerra (MA 304337).

Spain: Ávila, Castronuevo, encinares adehesados, 19-VI-1984, Barrera *et al.* (MA 477392); Badajoz, Campanario, VI-1911, Lagares (MA 109442); Mérida, 23-IV-1986, Fernández Díez (MA 477220); Quintana de la Serena, 11-VI-1971, Casaseca (MA 191645); Burgos, Carazo, camino de la ermita de la Virgen del Sol, 30VM7047, 1100 m, 12-VII-1979, Pons-Sorolla & Susanna (MA 414158); Oña, claros de pinares, 650 m, 30TVN6325, 27-VI-1987, Gil Zúñiga & Alejandre (MA 423614); Ciudad Real, Piedrabuena, 1 km después de Tabla de la Yedra, hacia "El Gargantón", 30SUJ9124, 570 m, 6-VI-1992, Castilla & Martín-Blanco (MA 627082); Guadalajara, Valdenuño, 30TVL6815, 900 m, matorrales sobre sustratos silíceos, 30-XII-1992, Garin (MA 563742); Huelva, Reserva Biológica de Doñana, limite con carretera de Matalascañas, Valdés (MA 427225); Sierra de Aracena, entre Valdeflores e Higuera de la Sierra, arroyo del Rey, 24-

II-1978, Rivera (MA 428833); Jaén, La Carolina, alrededores olivar, terreno silíceo, 600 m 30SVH4737, 30-V-1988, Payer (MA 554201); Madrid, Cercedilla, Sierra de Guadarrama, Vicioso (MA 109463); Chapinería, 30TUK97, 21-IV-1990, Giráldez (SALA 106305); Dehesa de Arganda, 30-IV-1966, Bellot & Monasterio (MA 633043); Orense, Cudeiro, en los alrededores de la capital, 15-XI-1987, Amigo (MA 478057); Salamanca, Castellanos de Villiquera, 7-VI-1967, Casaseca (MA 191679); Segovia, Brieva, limítrofe con la Higuera, 30TVL1043, 1020 m pastos rocosos silíceos, 21-V-1988, García Ada (MA 560127); Pedraza, carretera de Aldealengua de Pedraza, 30TVL3251, 1100 m, sabinar adehesado para pasto, 6-VI-1987, García Ada (MA 560109); Revenga, 30TVL0724, 1160 m pastos y prados silíceos próximos al embalse, 11-VI-1988, García Ada (MA 560128); Valladolid, Castronuño, Dehesa de Cubillas, 14-VII-1988, Valle & Balbás (SALA 23890).

Linaria viscosa (L.) Chaz. **subsp. *viscosa***

Portugal: Baixo Alentejo, s.r., 10-IV-1946, Rainha (MA 109351); Cabo de Sines, 25-V-1978, Devesa *et al.* (SEV 39947)**.**
Spain: Badajoz, Calamonte, 300 m, UTM 29SDQ20, 9-II-1982, Castroviejo (SALA 14341); La Albuera, 300 m, 14-IV-1976, Segura Zubizarreta (SALA 8778); Villanueva de la Serena, 11-II-1984, Ladero *et al.* (SALA 6721); Villanueva de la Serena, 19-III-1980, Ladero & Pérez Chiscano (SALA 11824); Cádiz, Chiclana, pinares, suelo arenoso, 9-III-1978, Pastor *et al.* (SEV 96988); San Roque, Sierra Carbonera, 300 m, 17-IV-1974, Talavera & Valdés (SEV 124907); Huelva, Bonares, 9-I-1978, Pastor *et al.* (SEV 30415); Entre Hinojos y Almonte, 8-IV-1978, Cabezudo (SEV 33007); Isla Cristina, arenas interiores playas de Lepe, 17-III-1967, Borja *et al.* (SALA 4809); Isla Cristina, arenas interiores playas de Lepe, 17-III-1968, Borja *et al.* (SALA 3207); Málaga, entre Grazalema y Ronda, 700 m, suelo arenoso, 23-V-1966, Getliffe *et al.* (SALA 3543); Sierra de Grazalema, Ronda, Los Alcornocales, 30STF9671, 760 m, areniscas, 8-VI-1993, Aparicio *et al.* (MA 527196); Murcia, Loma rasa próxima a Alcaraz, 27-VI-1923, Cuatrecasas (MA 109347); Sevilla, Alcalá de Guadaira, carretera del Arahal, 1-IV-1980, Luque *et al.* (SEV 96532); Bollullos de la Mitación, 10-III-1983, Diez *et al.* (SEV 96659); Mairena de Alcor, 5-IV-1975, Domínguez *et al.* (SEV 96521); Puebla del Río, cerca de la Isla Mayor, suelo arenoso ácido, 17-III-1968, Galiano *et al.* (SALA 3542); Valencia, Carcaixent, YJ22, 200 m, 24-IV-1986, Mateo *et al.* (MA 383730); Carcaixent, YJ22, 200 m, 24-IV-1986, Mateo *et al.* (MA 388879).

Linaria viscosa* subsp. *spicata (Coutinho) D.A. Sutton

Spain: Granada, Albuñuelas, Los Becardes, 30SVF3587, 1320 m, 10-VI-1977, Muñoz Garmendia (MA 208192); Dientes de la Vieja, 9-VI-1927, Lacaita (MA 109363); Sierra de Almijara, Sierra del Chaparral, 1200 m, 30-VI-1966, Getliffe *et al.* (MA 194883); Sierra Tejeda, Venta de Zafarraya, 28-V-1931, Ceballos (MA 109576); Jaén, Segura de la Sierra, Sierra de las Cuatro Villas, Puerto de Beas, 30SWH2136, 1000 m, 6-VI-1980, s.r. (MA 512713); Málaga, Canillas de Albaida, 30-V-1931, Ceballos (MA 109577); Frigiliana, 30-V-1931, Vicioso (MA 429631); Manilva, 9-V-1932, Vicioso (MA 109330); Sierra Tejeda, Puerto de la Horza, VI-1913, Gros (MA 109355); Sierra Tejeda al Puerto de la Horza, VI-1913, Gros (MA 109361); s.r., 8-VI-1934, Cuatrecasas (MA 109362).

Table S1. Specimens sampled for sequencing of DNA regions. Voucher specimens are indicated.

Taxon	(Population code) Sampled locality	Voucher
Antirrhinum L.		
Antirrhinum graniticum Rothm.	Spain, Madrid, Fuentidueña del Tajo	VAL 99540
Chaenorhinum (DC.) Rchb.		
Chaenorhinum minus (L.) Lange	Unknown	McNeils 96-336 (GH)
Linaria Mill.		
Linaria sect. *Diffusae* (Benth.) Wettst.		
L. reflexa (L.) Chaz.	Algeria, Algiers	J.J. Aldasoro A9799 (MA)
Linaria sect. *Linaria*		
L. vulgaris Mill.	France, Chamonix	B. Estébanez s.n. (MA)
Linaria sect. *Macrocentrum* D.A.Sutton		
L. chalepensis (L.) Mill.	Cyprus, Larnaca, Cape Kiti	Iter Mediterraneum IV 294 (MA)
Linaria sect. *Pelisserianae* Valdés		
L. triornithophora (L.) Willd.	Spain, Cáceres, Puerto de Perales	M. Fernández-Mazuecos 18MF07 (MA)
Linaria sect. *Speciosae* (Benth.) Wettst.		
L. genistifolia (L.) Mill.	Turkey, Hadim-Bezkir	J.J. Aldasoro & M.L. Alarcón A9751 (MA)
L. repens (L.) Mill.	Spain, Cuencia, Beteta	M. Fernández-Mazuecos 54MF09 (MA)
Linaria sect. *Supinae* (Benth.) Wettst.		
L. alpina (L.) Mill.	Spain, Huesca, Bujaruelo	J. Güemes s.n. (MA)
L. amoi Campo ex Amo	Spain, Málaga, Cómpeta	M. Fernández-Mazuecos, A.D. Forrest & P. Vargas 30PV08 (MA)
L. micrantha (Cav.) Hoffmanns. & Link	Spain, Alicante, Vall de Gallinera	J.X. Soler & M. Signes 1530-JXS (MA)
Linaria sect. *Versicolores* (Benth.) Wettst.		
Subsect. *Versicolores*		
L. algarviana Chav.	Portugal, Cabo de São Vicente	M. Fernández-Mazuecos 11MF09 (MA)
L. bipartita (Vent.) Willd.	Morocco, Rabat	S.L. Jury with R.G. Wilson 18558 (RNG)
L. bordiana Santa & Simonneau	Algeria, Sidi Lakhdar	D.A. & S.J. Sutton 172 (RNG)
L. clementei Haensel. ex Boiss.	Spain, Málaga, Alhaurín de la Torre	M. Fernández-Mazuecos, A.D. Forrest & P. Vargas 7MF08 (MA)
L. gharbensis Batt. & Pit.	Spain, Huelva, Gibraleón	M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 7MF09 (MA)
L. hellenica Turrill	Greece, Kambos	Unknown collector (ATH)
L. imzica Gómiz	Morocco, Jbel Imzi	F. Gómiz s.n. (MA)
L. incarnata (Vent.) Spreng.	(M1) Morocco, Kenitra-Khemisset	S. Martín-Bravo, I. Pulgar, F.J. Fernández, G.C. Mazo 34SMB06 (MA)
L. incarnata (Vent.) Spreng.	(M2) Morocco, Salé	J. Lambinon & G. van den Sande nº95/Ma/333 (RNG)
L. incarnata (Vent.) Spreng.	(I1) Spain, Badajoz, Alburquerque	M. Fernández-Mazuecos 9MF09 (MA)
L. incarnata (Vent.) Spreng.	(I2) Spain, Cáceres, Torrejón el Rubio	J.L. Pérez Chiscano s.n. (RNG)
L. incarnata (Vent.) Spreng.	(I3) Spain, Zamora, Bercianos de Aliste	B. Estébanez & N. García s.n. (MA)
L. incarnata (Vent.) Spreng.	(I4) Spain, Salamanca, Pelabravo	M. Fernández-Mazuecos & P. Vargas 39MF09 (MA)

Table S1. Continued

L. incarnata (Vent.) Spreng.	(I5) Spain, Cáceres, Valencia de Alcántara	M. Fernández-Mazuecos 62MF10 (MA)
L. incarnata (Vent.) Spreng. (= *L. onubensis* Pau)	(O1) Spain, Huelva, Valverde del Camino	V. Valcarcel & P. Vargas 5PV08 (MA)
L. incarnata (Vent.) Spreng. (= *L. onubensis* Pau)	(O2) Spain, Huelva, Fuente de la Corcha	M. Fernández-Mazuecos & A. Bañón 28MF10 (MA)
L. incarnata (Vent.) Spreng. (= *L. onubensis* Pau)	(O3) Spain, Huelva, Niebla	E. Sánchez-Gullón s.n. (MA)
L. maroccana Hook.f.	Morocco, Marrakech – Tizi-n-Test	S.L. Jury, B. Tahiri & T.M. Upson 14209 (RNG)
L. multicaulis (L.) Mill. subsp. multicaulis	Italy, Sicily, Etna	I. Álvarez *et al.* IA1622 (MA)
L. multicaulis subsp. *heterophylla* (Desf.) D.A.Sutton	Morocco, Azrou	M. Fernández-Mazuecos & J.C. Moreno 15MF08 (MA)
L. pedunculata (L.) Chaz.	Spain, Huelva, Marismas del Odiel	M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 4MF09 (MA)
L. pseudoviscosa Murb.	Tunisia, El Haouaria	P. Wilkin & E.J. Wellens 231 (RNG)
L. salzmannii Boiss.	Spain, Málaga, El Chorro	M. Fernández-Mazuecos & J. Ramírez 19MF09 (MA)
L. spartea (L.) Chaz.	(1) Spain, Madrid, Colmenar	P. Vargas 101PV07 (MA)
L. spartea (L.) Chaz.	(2) Spain, Soria, Tardelcuende	M. Fernández-Mazuecos, A. Quiroga, S.C. Herrera & D. Orgaz 14MF07 (MA)
L. tenuis (Viv.) Spreng.	Libya, Tripoli	Davis & Boulos 50581 (RNG)
L. tingitana Boiss. & Reut.	Algeria, El Macta	D.A. & S.J. Sutton 383 (RNG)
L. viscosa (L.) Chaz. subsp. *viscosa*	(1) Spain, Huelva, Marismas del Odiel	M. Fernández-Mazuecos, J.L. Blanco & E. Sánchez-Gullón 6MF09 (MA)
L. viscosa (L.) Chaz. subsp. *viscosa*	(2) Spain, Huelva, Matalascañas	M. Fernández-Mazuecos & J.L. Blanco 1MF09 (MA)
L. viscosa subsp. *spicata* (Coutinho) D.A.Sutton	(1) Spain, Jaén, Cazorla	M. Fernández-Mazuecos 49MF09 (MA)
L. viscosa subsp. *spicata* (Coutinho) D.A.Sutton	(2) Spain, Málaga, Cómpeta	M. Fernández-Mazuecos, A.D. Forrest & P. Vargas 9MF08 (MA)
L. weilleri Emb. & Maire	Morocco, Tirhmi	Miller, Russell & Sutton s.n. (RNG)
Subsect. Elegantes (Viano) D.A.Sutton		
L. elegans Cav.	Spain, Ávila, Plataforma de Gredos	E. Amat s.n. (MA)
L. nigricans Lange	Spain, Almería, Tabernas	P. Vargas 3PV08 (MA)

Apéndice 4

Análisis filogeográfico de una clado ibérico de *Linaria* sect. *Versicolores* basado en secuencias del ADN plastidial

Manuscrito inédito

Introducción

En el análisis filogenético de *Linaria* sect. *Versicolores* basado en dos regiones del ADN plastidial (*rpl*32-*trn*L^{UAG} y *trn*K-*mat*K), se detectaron cuatro clados principales (véase Fig. 1 del Capítulo 3; Fernández-Mazuecos & Vargas, 2011). Uno de ellos (denominado "clado II", en adelante "clado ibérico") estuvo constituido por una serie de táxones endémicos o subendémicos de la península Ibérica: *L. algarviana*, *L. clementei*, *L. incarnata*, *L. onubensis*, *L. salzmannii*, *L. spartea*, *L. viscosa* subsp. *spicata* y *L. viscosa* subsp. *viscosa* (véase el Apéndice 3 para una discusión acerca de la aceptación de *L. onubensis* y de la nueva delimitación de *L. incarnata*). Dentro de este clado se obtuvo escasa resolución, aunque sí se detectó un linaje bien apoyado formado por táxones del sureste ibérico: *L. clementei*, *L. salzmannii* y *L. viscosa* subsp. *spicata*. La adición de una región más (*trn*S-*trn*G) a la filogenia plastidial de *Versicolores* apenas resultó en un aumento de resolución para este clado (véase Fig. 3B del Capítulo 4). Aunque el análisis conjunto de las tres regiones plastidiales y una nuclear (ITS) confirmó la monofilia del clado ibérico (véase Fig. 4 del Capítulo 4), las relaciones dentro del mismo permanecieron escasamente resueltas. Posteriormente, en el contexto del estudio de la polifilia de *L. incarnata* (*sensu* Viano, 1969), se efectuó un análisis filogenético basado en tres marcadores plastidiales: *rpl*32-*trn*L^{UAG}, *trn*S-*trn*G y un fragmento de *ndh*F (la región *trn*K-*mat*K no se empleó por la práctica ausencia de variabilidad dentro del clado ibérico) (Apéndice 3). En este caso se recuperó una mayor resolución, con dos clados hermanos bien apoyados, uno formado por *L. clementei*, *L. salzmannii* y *L. viscosa* subsp. *spicata* y otro por *L. incarnata*, *L. algarviana*, *L. spartea* y *L. viscosa* subsp. *viscosa* (véase Fig. 5B del Apéndice 3). Asimismo, dentro del segundo se obtuvo un subclado bien apoyado, y se encontró un cierto grado de variabilidad nucleotídica dentro de los dos linajes principales. Aun así, dada la baja resolución de los árboles filogenéticos, una aproximación basada en redes de haplotipos es probablemente más adecuada para analizar la variabilidad de secuencias plastidiales del clado ibérico.

En este anexo se presenta un análisis filogeográfico preliminar del linaje ibérico, basado en secuencias plastidiales de las tres regiones que han proporcionado una mayor variabilidad y resolución filogenética en estudios anteriores (*rpl*32-*trn*L^{UAG}, *trn*S-*trn*G y *ndh*F). Para ello, se ha efectuado un muestreo representativo de poblaciones de todos los táxones (con especial incidencia en los de amplia distribución), y se han reconstruido las relaciones filogenéticas (mediante árboles filogenéticos y red de haplotipos) entre las secuencias plastidiales de un

individuo de cada población. El objetivo es explorar las relaciones filogenéticas y los patrones filogeográficos del clado ibérico.

Material y métodos

Estrategia de muestreo y secuenciación de ADN

Se muestreó un total de 49 individuos pertenecientes a otras tantas poblaciones (32 de ellas no incluidas en estudios anteriores; Tabla 1). El número de poblaciones muestreadas por especie dependió de la amplitud de su área de distribución, así como de la disponibilidad de material: una población de *L. algarviana*; una de *L. salzmannii*; dos de *L. clementei*; tres de *L. onubensis*; cinco de *L. incarnata*; siete de *L. viscosa* subsp. *spicata*; 14 de *L. viscosa* subsp. *viscosa*; y 15 de *L. spartea*. Además, se incluyó por primera vez un individuo correspondiente a *L. viscosa* subsp. *crassifolia*. Aunque la identidad de este taxon es dudosa (no fue aceptado en la última revisión del género *Linaria* para la península Ibérica; Sáez & Bernal, 2009), el individuo se recolectó en la localidad tipo de la subespecie, donde parece ser una planta extremadamente rara (L. Sáez, comunicación personal).

Para la extracción de ADN y la secuenciación de las tres regiones plastidiales (*rpl*32-*trn*L^{UAG}, *trn*S-*trn*G y el fragmento final de *ndh*F) se siguieron los protocolos descritos por Fernández-Mazuecos & Vargas (2011; Capítulo 3) y Vigalondo *et al.* (en preparación; Apéndice 3). Además de los individuos muestreados del clado ibérico, se obtuvieron secuencias de una muestra representativa de especies del resto de clados de la sección *Versicolores*, así como de dos especies de otras secciones de *Linaria* (*L. chalepensis* y *L. vulgaris*) (Tabla 1).

Análisis de secuencias

Tras alinearse las secuencias (Katoh *et al.*, 2002), se concatenaron las tres regiones en una única matriz, y ésta se analizó mediante técnicas de reconstrucción de árboles filogenéticos: inferencia bayesiana (IB), máxima verosimilitud (MV) y máxima parsimonia (MP) (Goloboff *et al.*, 2003; Guindon & Gascuel, 2003; Ronquist & Huelsenbeck, 2003). *L. chalepensis* se empleó como grupo externo. Asimismo, se obtuvo una red de haplotipos a partir de las secuencias del clado ibérico mediante parsimonia estadística (Templeton *et al.*, 1992; Clement *et al.*, 2000). En

Tabla 1. Especímenes de *Linaria* sect. *Versicolores* y el grupo externo muestreados y utilizados para la secuenciación de regiones del ADN plastidial (*rpl*32-*trn*L^{UAG}, *trn*S-*trn*G y *ndh*F). Se indican las localidades y referencias de las recolecciones.

Taxon	(Nº de población) Localidad	Recolección
Linaria Mill.		
Linaria sect. *Linaria*		
L. vulgaris Mill.	Francia, Chamonix	B. Estébanez s.n. (MA)
Linaria sect. *Macrocentrum* D.A.Sutton		
L. chalepensis (L.) Mill.	Chipre, Larnaca, Cape Kiti	Iter Mediterraneum IV 294 (MA)
Linaria sect. *Versicolores* (Benth.) Wettst.		
L. algarviana Chav.	Portugal, Cabo de São Vicente	M. Fernández-Mazuecos 11MF09 (MA)
L. clementei Haensel. ex Boiss.	España, Málaga, Alhaurín de la Torre	M. Fernández-Mazuecos, A.D. Forrest & P. Vargas 7MF08 (MA)
L. clementei Haensel. ex Boiss.	España, Málaga, Coín	M. Fernández-Mazuecos & J. Ramírez 24MF09 (MA)
L. elegans Cav.	España, Ávila, Plataforma de Gredos	E. Amat s.n. (MA)
L. gharbensis Batt. & Pit.	España, Huelva, Gibraleón	M. Fernández-Mazuecos *et al.* (MA)
L. imzica Gómiz	Marruecos, Jbel Imzi	F. Gómiz s.n. (MA)
L. incarnata (Vent.) Spreng.	España, Zamora, Bercianos de Aliste	B. Estébanez & N. García s.n. (MA)
L. incarnata (Vent.) Spreng.	España, Salamanca, Pelabravo	M. Fernández-Mazuecos & P. Vargas 39MF09 (MA)
L. incarnata (Vent.) Spreng.	España, Badajoz, Alburquerque	M. Fernández-Mazuecos 9MF09 (MA)
L. incarnata (Vent.) Spreng.	España, Cáceres, Torrejón el Rubio	J.L. Pérez Chiscano s.n. (RNG)
L. incarnata (Vent.) Spreng.	España, Cáceres, Valencia de Alcántara	F. Conti *et al.* s.n. (RNG)
L. mamorensis Mazuecos, Vigalondo & L.Sáez	Marruecos, Kenitra-Khemisset	S. Martín-Bravo *et al.* 34SMB06 (MA)
L. maroccana Hook.f.	Marruecos, Marrakech – Tizi-n-Test	S.L. Jury, B. Tahiri & T.M. Upson 14209 (RNG)
L. multicaulis subsp. *heterophylla* (Desf.) D.A.Sutton	Marruecos, Azrou	M. Fernández-Mazuecos & J.C. Moreno 15MF08 (MA)
L. nigricans Lange	España, Almería, Tabernas	P. Vargas 3PV08 (MA)
L. onubensis Pau	España, Huelva, Valverde del Camino	V. Valcarcel & P. Vargas 5PV08 (MA)
L. onubensis Pau	España, Huelva, Niebla	E. Sánchez-Gullón s.n. (MA)
L. onubensis Pau	España, Huelva, Fuente de la Corcha	M. Fernández-Mazuecos & A. Bañón 28MF10 (MA)
L. pedunculata (L.) Chaz.	España, Huelva, Marismas del Odiel	M. Fernández-Mazuecos *et al.* 4MF09 (MA)
L. salzmannii Boiss.	España, Málaga, El Chorro	M. Fernández-Mazuecos & J. Ramírez 19MF09 (MA)
L. spartea (L.) Chaz.	España, León, Villalís de la Valduerna	B. Estébanez & N. Medina s.n. (MA)
L. spartea (L.) Chaz.	España, León, Alija del Infantado	B. Estébanez & N. Medina s.n. (MA)
L. spartea (L.) Chaz.	España, Zamora, Fonfría	B. Estébanez & N. Medina s.n. (MA)
L. spartea (L.) Chaz.	España, Soria, Tardelcuende	M. Fernández-Mazuecos *et al.* 14MF07 (MA)
L. spartea (L.) Chaz.	España, Madrid, Colmenar Viejo	P. Vargas 101PV07 (MA)
L. spartea (L.) Chaz.	España, Sa, Arapiles	M. Fernández-Mazuecos & P. Vargas 40MF09 (MA)
L. spartea (L.) Chaz.	España, Toledo, Velada	M. Fernández-Mazuecos 3MF08 (MA)

Tabla 1. Continuación.

L. spartea (L.) Chaz.	España, Ciudad Real, Solana del Pino	MA 596688
L. spartea (L.) Chaz.	España, Cáceres, Monfragüe	M. Fernández-Mazuecos 4MF08 (MA)
L. spartea (L.) Chaz.	España, Badajoz, Aljucén	Carmen E. González 1CGC (UPOS)
L. spartea (L.) Chaz.	España, Badajoz, Alburquerque	M. Fernández-Mazuecos 10MF09 (MA)
L. spartea (L.) Chaz.	Portugal, Montemor-o-Novo	M. Fernández-Mazuecos 18MF09 (MA)
L. spartea (L.) Chaz.	Portugal, Praia da Adraga	M. Fernández-Mazuecos 13MF09 (MA)
L. spartea (L.) Chaz.	España, Huelva, El Romerano	E. Sánchez-Gullón s.n. (MA)
L. spartea (L.) Chaz.	España, Huelva, Marismas del Odiel	M. Fernández-Mazuecos *et al.* 5MF09 (MA)
L. viscosa subsp. *crassifolia* (Coutinho) D.A.Sutton	Portugal, Cabo da Roca	L. Sáez (BCB)
L. viscosa subsp. *spicata* (Coutinho) D.A.Sutton	España, Málaga, Cómpeta	M. Fernández-Mazuecos *et al.* 9MF08 (MA)
L. viscosa subsp. *spicata* (Coutinho) D.A.Sutton	España, Málaga, Canillas de Albaida	M. Fernández-Mazuecos *et al.* 10MF08 (MA)
L. viscosa subsp. *spicata* (Coutinho) D.A.Sutton	España, Málaga, Canillas del Aceituno	M. Fernández-Mazuecos & J.L. Blanco 28MF09 (MA)
L. viscosa subsp. *spicata* (Coutinho) D.A.Sutton	España, Granada, Albuñuelas	F. Muñoz Garmendia 1902 (RNG)
L. viscosa subsp. *spicata* (Coutinho) D.A.Sutton	España, Granada, Sierra Nevada	J.L. Blanco-Pastor 147JB10 (MA)
L. viscosa subsp. *spicata* (Coutinho) D.A.Sutton	España, Jaén, Cazorla	M. Fernández-Mazuecos 49MF09 (MA)
L. viscosa subsp. *spicata* (Coutinho) D.A.Sutton	España, Jaén, Villanueva del Arzobispo	M. Fernández-Mazuecos 45MF09 (MA)
L. viscosa (L.) Chaz. subsp. *viscosa*	Portugal, Sines	Barbosa, Gomes & Moreno s.n. (RNG)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Huelva, Isla Cristina	E. Sánchez-Gullón s.n. (MA)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Huelva, Mazagón	E. Sánchez-Gullón s.n. (MA)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Huelva, Lucena del Puerto	E. Sánchez-Gullón s.n. (MA)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Huelva, Matalascañas	M. Fernández-Mazuecos & J.L. Blanco 1MF09 (MA)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Sevilla, Puebla del Río - Aznalcázar	V.H. Heywood *et al.* 385 (RNG)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Sevilla, El Arahal - Osuna	D. Bramwell *et al.* s.n. (RNG)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Cádiz, Chipiona	J. Ruano Martínez 12JRM (UPOS)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Cádiz, Puerto de Santa María	J.L. Blanco-Pastor 11JLB09 (MA)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Cádiz, Arcos de la Frontera-Bornos	E.A. Leadlay *et al.* s.n. (RNG)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Cádiz, Alcalá de los Gazules	B. Cabezudo *et al.* 278/71 (RNG)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Cádiz, Zahara de los Atunes	C.R. Fraser-Jenkins s.n. (RNG)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Cádiz, Castellar - Almoraima	V.H. Heywood *et al.* 617 (RNG)
L. viscosa (L.) Chaz. subsp. *viscosa*	España, Cádiz, Zahora	E. Sánchez-Gullón s.n. (MA)

todos los casos, se utilizaron los métodos y programas informáticos descritos por Fernández-Mazuecos & Vargas (2011).

Resultados

Los tres análisis filogenéticos produjeron resultados congruentes (Fig. 1). La monofilia del clado ibérico recibió un alto apoyo en todos los análisis (probabilidad posterior bayesiana, PP = 1; *bootstrap* de MV, BS-MV = 100%; *bootstrap* de MP, BS-MP = 98%). Dentro del clado ibérico se obtuvieron dos grupos monofiléticos hermanos bien apoyados. Todos los individuos secuenciados de *L. onubensis*, *L. algarviana*, *L. spartea*, *L. incarnata* y *L. viscosa* subsp. *crassifolia* se incluyeron en el primero ("linaje 1", Figs. 1, 2; PP = 1; BS-MV = 87%; BS-MP = 81%), mientras que todos los individuos de *L. clementei*, *L. salzmannii* y *L. viscosa* subsp. *spicata* se incluyeron en el segundo ("linaje 2", Figs. 1, 2; PP = 1; BS-MV = 95%; BS-MP = 94%). Los individuos de *L. viscosa* subsp. *viscosa* se repartieron entre ambos linajes. Dentro de los linajes 1 y 2 se obtuvo escasa resolución, a excepción de tres clados bien apoyados por el análisis bayesiano (Fig. 1). En la red de haplotipos (Fig. 2A, B), los linajes 1 y 2 quedaron separados por cinco sustituciones nucleotídicas y cuatro haplotipos ausentes (extintos o no muestreados) (Fig. 2B).

Los linajes de haplotipos tuvieron una escasa correspondencia con la delimitación taxonómica de las especies y subespecies. En particular, los individuos de *L. viscosa* subsp. *viscosa* dieron ocho haplotipos distintos ampliamente distribuidos por toda la red. Por el contrario, para otros táxones en los que secuenció más de un individuo, se obtuvo un reducido número (1-3) de haplotipos estrechamente emparentados (*L. onubensis*, *L. incarnata*, *L. viscosa* subsp. *spicata*). Sin embargo, se detectó una clara estructuración geográfica de los linajes de haplotipos (Fig. 2A). La distribución del linaje 2 se restringió al sureste de la península Ibérica, principalmente a zonas de altitud media o elevada de las cordilleras Béticas (Serranía de Ronda, Sierra de Mijas, Sierra de Tejeda, Sierra de Almijara, Sierra Nevada y Sierra de Cazorla). En el límite occidental de la distribución del linaje se encontraron individuos a baja altitud (identificados como *L. viscosa* subsp. *viscosa*, Fig. 2). El linaje 1 estuvo distribuido por el resto de la península Ibérica (Fig. 2A). Es llamativa la ausencia de solapamiento de la distribución de ambos linajes en la zona en que contactan (provincia de Cádiz, Fig. 2A).

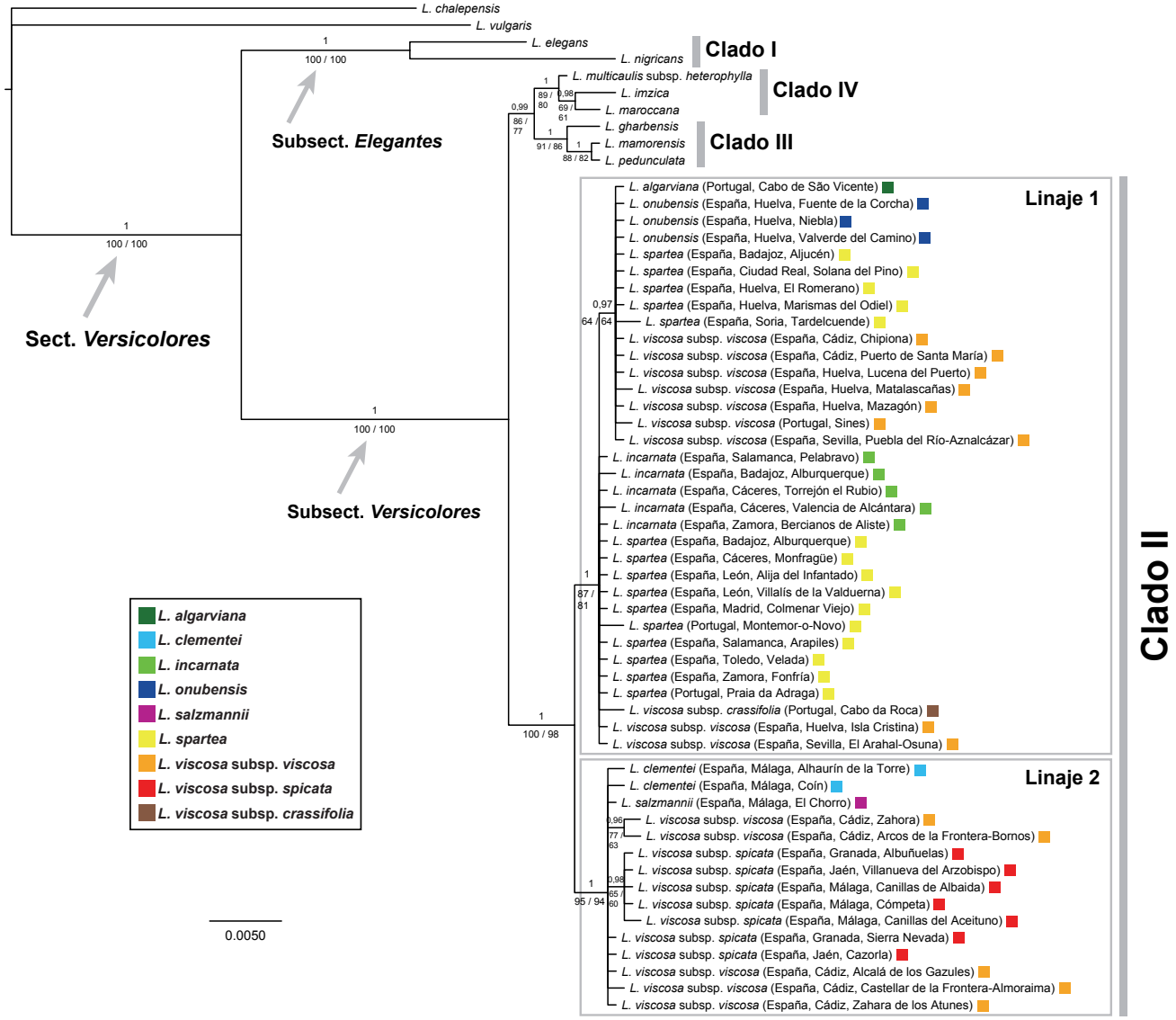


Fig. 1. Relaciones filogenéticas, basadas en el análisis combinado de tres regiones del ADN plastidial (*rpl*32-*trn*L^{UAG}, *trn*S-*trn*G y *ndh*F), entre 49 individuos pertenecientes a las nueve especies y subespecies del clado ibérico de *Linaria* sect. *Versicolores*. Se muestra el árbol consenso (obtenido por la regla de la mayoría del 50%) resultante del análisis bayesiano. Los números sobre las ramas son las probabilidades posteriores bayesianas. Los números bajo las ramas son los valores de *bootstrap* de máxima verosimilitud / máxima parsimonia. Se indican los linajes discutidos en el texto.

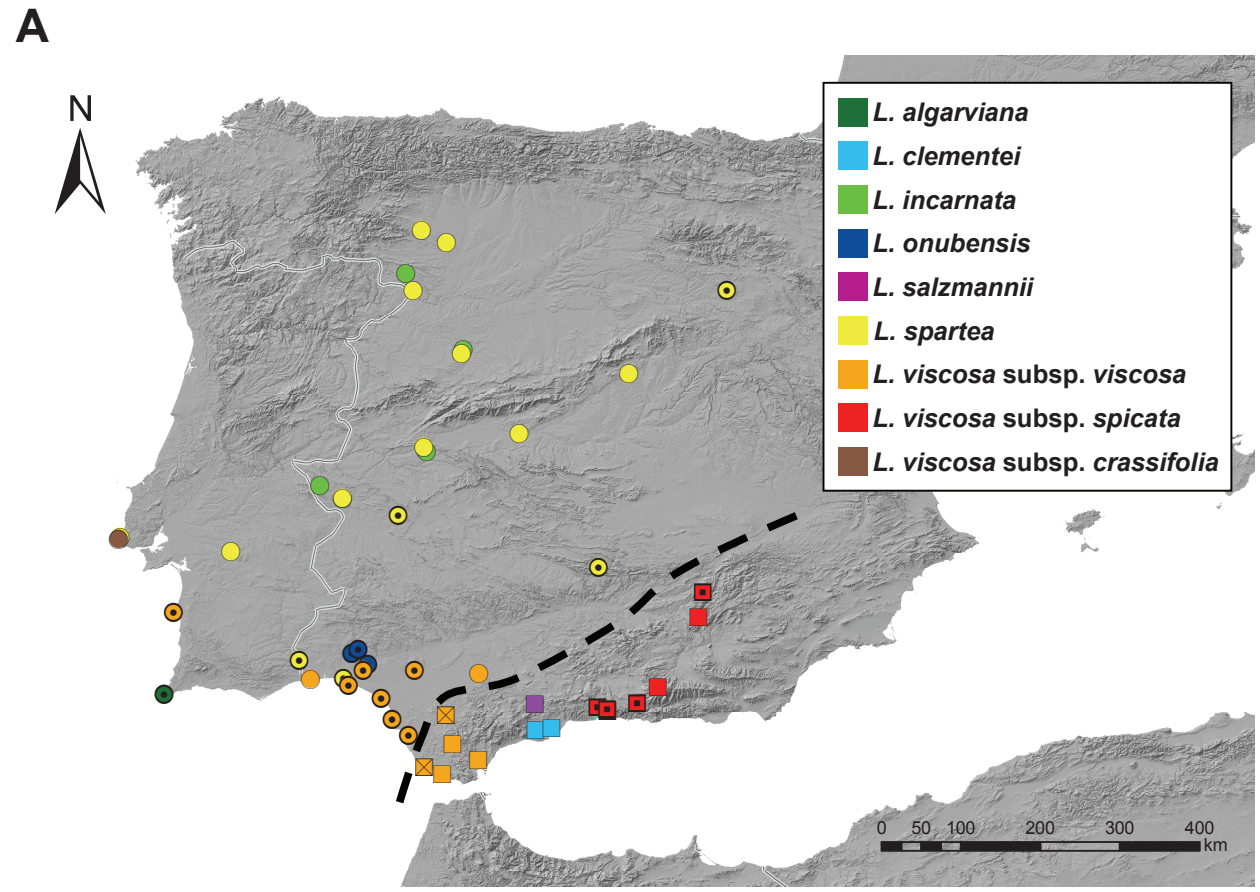
Discusión

Los presentes resultados reafirman la monofilia del clado ibérico obtenida en estudios anteriores a partir de secuencias plastidiales de *Linaria* sect. *Versicolores* (Capítulos 3 y 4; Apéndice 3; Fernández-Mazuecos & Vargas, 2011) y del análisis combinado de secuencias plastidiales y nucleares (Capítulo 4). También se reafirma la monofilia de los dos linajes hermanos que constituirían el clado ibérico, ya sugeridos en los estudios citados con apoyo variable. En particular, en el análisis combinado de secuencias nucleares y plastidiales de Fernández-Mazuecos *et al.* (Fig. 4 del Capítulo 4), el linaje 2 recibió alto apoyo (PP = 1), mientras que para el linaje 1 se obtuvo un apoyo más bajo (PP = 0.77). La relativamente reducida variabilidad de secuencias encontrada en el presente estudio (17 haplotipos en un complejo de 8-9 táxones, frente a los 20 haplotipos encontrados sólo en *L. elegans*, Capítulo 5) es congruente con la reciente y rápida diversificación del clado (Fig. 4 del Capítulo 4): en el Capítulo 4 se estimó la edad de la divergencia entre los linajes 1 y 2 en un máximo de c. 740.000 años. La clara estructura filogeográfica encontrada (Fig. 2A) sugiere un notable aislamiento geográfico entre los linajes desde entonces. Una diferenciación geográfica similar entre las poblaciones béticas y las del resto de la península Ibérica se ha encontrado en *Hypochaeris radicata* (Ortiz *et al.*, 2009). Este patrón de diferenciación podría explicarse, en principio, por el papel de barrera biogeográfica ejercido por la cuenca del Guadalquivir, o por el antiguo Golfo Tartésico que ocupaba su lugar. Sin embargo, la diversificación del clado ibérico de *Linaria* sect. *Versicolores* parece ser posterior al rellenado de la cuenca (Plioceno; Meléndez Hevia, 2004), con lo que el brazo de mar que constituía el Golfo Tartésico no habría tenido un papel en el aislamiento entre los dos linajes. Tampoco parece que la cuenca, en su configuración actual, suponga una barrera para la dispersión de semillas, teniendo en cuenta que al menos uno de los táxones (*L. viscosa* subsp. *viscosa*) se encuentra ampliamente distribuido por las zonas bajas de la misma. Por el contrario, es posible que la especialización ecológica, en particular la asociada al tipo de sustrato, haya tenido un importante papel en el aislamiento de los linajes 1 y 2. En efecto, el linaje 1 parece estar asociado a sustratos ácidos (silíceos o arcillosos), mientras que el linaje 2 se asocia a los sustratos básicos (calizos o dolomíticos) predominantes en las Cordilleras Béticas. Esta diferenciación de linajes asociados a distintos sustratos es similar a la encontrada en *Carex* sect. *Spirostachyae* (Escudero *et al.*, 2008).

La ausencia de monofilia recíproca de las especies y subespecies del clado ibérico es fácilmente explicable en el marco del proceso de coalescencia (Knowles & Carstens, 2007). La "repartición incompleta de linajes" (más conocida por el término en inglés *incomplete lineage sorting*) es, de hecho, esperable en un linaje de diversificación rápida, y con tamaños efectivos poblacionales presumiblemente elevados en algunos casos (particularmente *L. spartea* y *L. viscosa* subsp. *viscosa*) (Carstens & Knowles, 2007; Knowles & Carstens, 2007). Además, no se puede descartar que procesos de hibridación también estén relacionados con las incongruencias entre el patrón filogeográfico y el morfológico (Willyard *et al.*, 2009). En el caso de *L. viscosa* subsp. *viscosa*, por ejemplo, los individuos de su núcleo principal de distribución se incluyen en el linaje 1, mientras que los de su límite oriental (provincia de Cádiz) se incluyen en el linaje 2. Aunque la morfología general de estos individuos orientales corresponde claramente a *L. viscosa* subsp. *viscosa*, algunos individuos presentan ciertos caracteres más típicos de *L. viscosa* subsp. *spicata* (linaje 2), tales como los pedicelos adnatos al eje de la inflorescencia (Sutton, 1988; Sáez & Bernal, 2009). Estos individuos intermedios sugieren la presencia de una posible zona híbrida (Hewitt, 1988; Jiggins & Mallet, 2000) producida por el contacto secundario de los linajes 1 y 2, en una región donde también contactan los sustratos característicos de los dos linajes.

Por otro lado, los presentes resultados ponen de manifiesto que la delimitación taxonómica de *L. viscosa* requiere más investigación. Ninguno de los análisis filogenéticos efectuados hasta el momento (Capítulos 3 y 4, este Apéndice) sugiere un estrecho parentesco entre las subespecies

Fig. 2. Análisis de haplotipos plastidiales del clado ibérico de *Linaria* sect. *Versicolores*, basado en la combinación de las regiones *rpl*32-*trn*L^{UAG}, *trn*S-*trn*G y *ndh*F. (A) Distribución geográfica de los individuos muestreados. Los colores representan especies y subespecies. Los símbolos representan grupos de haplotipos, tal y como se delimitan en B. La línea discontinua indica el límite entre las distribuciones de los linajes 1 y 2. (B) Red de haplotipos obtenida mediante parsimonia estadística. Cada haplotipo encontrado se representa por un círculo de tamaño proporcional al número de individuos en los que se encontró. Los sectores coloreados representan la proporción de individuos de las distintas especies y subespecies en las que se encontró cada haplotipo. Las líneas indican sustituciones nucleotídicas, y los puntos negros representan haplotipos ausentes (extintos o no muestreados). Se delimitan los dos linajes principales discutidos en el texto, así como los grupos de haplotipos representados en A.


A
N
L. algarviana
L. clementei
L. incarnata
L. onubensis
L. salzmannii
L. spartea
L. viscosa subsp. viscosa
L. viscosa subsp. spicata
L. viscosa subsp. crassifolia
0 50 100 200 300 400
km
B
Lineage 1
Lineage 2

viscosa y *spicata*, o al menos no un parentesco mayor entre ellas que con el resto de las especies del clado. Por tanto, es posible que un estudio morfológico y molecular más profundo venga a confirmar que se trata, en realidad, de dos especies distintas: *L. viscosa* (L.) Chaz. y *L. spicata* Kunze. Por otro lado, la subsp. *crassifolia* es probablemente asimilable a la *L. viscosa* típica (Sáez & Bernal, 2009).

La resolución a escala detallada de las relaciones filogenéticas dentro del linaje ibérico requerirá la secuenciación de un mayor número de regiones de ADN, en particular del genoma nuclear. Además, la reciente y rápida especiación del grupo, probablemente acompañada de hibridación y repartición incompleta de linajes, hará necesaria la aplicación de nuevos métodos filogenéticos basados en la teoría de la coalescencia, que tienen en cuenta tales procesos (Liu, 2008; Maureira-Butler *et al.*, 2008; Edwards, 2009; Heled & Drummond, 2010; Blanco-Pastor *et al.*, 2012). Sólo entonces será posible una aproximación pormenorizada a los procesos de especiación del clado ibérico de *Linaria* sect. *Versicolores*.

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